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The current project is to study a previously identified breast cancer specific gene, originally designated BCSG1, in human breast cancer progression leading to metastasis. BCSG1, expressed in high abundance in metastatic breast cancer cDNA library but scarcely in normal breast cDNA library, was identified and cloned by differential cDNA sequencing. Interestingly, BCSG1 revealed no homology to any other known oncogenes; rather, BCSG1 revealed extensive sequence homology to Alzheimer disease (AD)-related neurotic proteins synuclein α (SNCA) and synuclein β (SNCB), and thus was also named as synuclein γ (SNCG). Preliminary studies demonstrated that: 1) SNCG expression was a stage-specific in human breast: undetectable in normal or benign breast lesions, low level and partial expression in low grade ductal carcinoma *in situ* but extremely high level in advanced infiltrating breast cancer; 2) SNCG expression in human breast cancer cells is dramatically suppressed by tumor growth inhibitor oncostatin M (OM), a cytokine predominantly produced by activated T cells and macrophages; 3) overexpression of SNCG in breast cancer cells led to a significant increase in cell motility and invasiveness *in vitro* and a profound augmentation of metastasis *in vivo*. The overall hypothesis to be evaluated is that SNCG, a neurotic protein mainly expressed in brain and localized to presynaptic terminals, play a critical role in breast cancer malignant progression from benign or non-invasive stage to metastatic stage. In this regard, up-regulation of SNCG will increase cell motility and thus facilitate invasion and metastasis.

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FOREWORD

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TABLE OF CONTENTS

I. ADJUSTMENT

The current Career Development Award was initially awarded to study the novel 80kDa matrix degrading proteinase in breast cancer progression. However, as we stated AND summarized in the last year's annual report, we have adjusted our proposed study to a complete new area studying a novel breast cancer related gene named as synuclein γ (SNCG).

II. INTRODUCTION

Identification of quantitative changes in gene expression that occur in the malignant mammary gland, if sufficiently characterized, may yield novel molecular markers which may be useful in the understanding of breast cancer development and progression (1). Within this context, we have previously reported the isolation of differentially expressed genes in the cDNA libraries from normal breast and infiltrating breast cancer using the EST-based differential cDNA sequencing approach (2, 3). Of many putative differentially expressed genes (2, 3), a breast cancer specific gene, BCSG1, which was (a) identified as a group of EST specifically expressed in mammary gland relative to other organs and was (b) high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified as a putative breast cancer specific gene (2).

Interestingly, BCSG1 revealed no homology to any other known growth factors or oncogenes; rather, BCSG1 revealed extensive sequence homology to AD-related neurotic proteins synucleins that are mainly expressed in brain and localized to presynaptic terminals (4-7). The pathological hallmark of AD is amyloid deposition in neurotic plaques and blood vessels (8). Two major intrinsic constituents of amyloid are a 39-43 AA peptide named Aß component (8) and the recently identified non-Aß component (NAC) (4). NAC precursor was cloned from a human brain library (4) and named synuclein α (SNCA) because it shares 95% sequence homology with rat synuclein. Recently, the second synuclein named synuclein ß (SNCB) was cloned from human brain and has 61% identical sequence with SNCA (6). The previously identified BCSG1, which is also highly expressed in brain (2), has 54% and 56% sequence identity with SNCA and SNCB, respectively, and has been renamed as synuclein γ (SNCG) (9). Thus, the previously unrecognized homology between these proteins defines a family of human brain synucleins that currently has three members. Although synucleins are abundant proteins expressed in presynaptic terminals and tightly associated with amyloid plaque in AD and Lewy body in Parkison's disease (PD) (10), their functions have not been defined yet. SNCA aggregation may be important in the etiology and pathogenesis in neurodegenerative disorders such as AD and PD (10). Being identified as a breast cancer specific gene, we previously demonstrated a stage-specific SNCG expression as follows: SNCG was undetectable in normal or benign breast lesions, showed partial expression in ductal carcinoma in situ, but was expressed at an extremely high level in advanced infiltrating breast cancer. The effects of SNCG on breast cancer growth and metastasis and the regulation of SNCG expression were also investigated.

III. BODY

III-1. Identification, cloning, and expression of SNCG (BCSG1) (Please see attached reprint 1 for detailed description)

Summary. A direct differential cDNA sequencing approach was employed to identify genes differentially expressed in normal breast as compared with breast cancer. Of many putative differentially expressed genes, a breast cancer specific gene SNCG, which was expressed in high

abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified as a putative breast cancer marker. *In situ* hybridization analysis demonstrated a stage-specific SNCG expression as follows: SNCG was undetectable in normal or benign breast lesions, showed partial expression in ductal carcinoma *in situ*, but was expressed at an extremely high level in advanced infiltrating breast cancer. The predicted amino acid sequence of SNCG gene has a significant sequence homology to non-Aß fragment of Alzheimer's disease amyloid protein. SNCG overexpression may indicate breast cancer malignant progression from benign breast or *in situ* carcinoma to the highly infiltrating carcinoma.

III-2. Stimulation of breast cancer invasion and metastasis by synuclein γ (SNCG) (see attached manuscript 1 in press)

Transfection of SNCG into MDA-MB-435 human breast cancer cells. In order to determine the effects of SNCG on tumorigenesis and invasion/metastasis, we have selected MDA-MB-435 human breast cancer cells as recipients for SNCG mediated gene transfection because of: 1) their lack of detectable of SNCG transcript (2); and 2) their highly tumorigenic and aggressive phenotype in nude mouse (11). Cells were transfected with a plasmid vector containing a neomycin resistance gene (neo clones), or the same vector containing a full-length of SNCG cDNA (SNCG clones). Clones were initially screened by *in situ* hybridization on slides with a specific SNCG antisense probe (data not shown), and the positive clones were subjected to Northern blot and Western blot analysis. MDA-MB-435 clones expressing SNCG were named as SNCG-435 clones, and the control neo transfected cells were named as neo-435 clones. Fig. 1 shows the Northern blot and Western blot analysis of SNCG expression in selected clones. All selected SNCG-435 clones expressed SNCG mRNA transcripts and proteins. In contrast, none of the neo-435 clones produced any detectable SNCG transcripts and proteins. No changes in morphology were observed in these clones. Based on the level of SNCG expression, we selected SNCG-435-1, SNCG-435-3, neo-435-1, and neo-435-2 clones for the subsequent studies.

In vitro growth of SNCG transfected cells. To determine whether SNCG overexpression affects the growth of MDA-MB-435 cells, cells from exponentially growing cultures of different MDA-MB-435 clones were seeded in triplicate at 3,000 cells per well (24-well plate) in 1 ml DMEM-5% serum. The growth rates of SNCG positive SNCG-435-1 and SNCG-435-3 cells were compared to that of SNCG negative neo-435-1 and neo-435-2 in a monolayer culture. No significant differences in growth rate were observed among SNCG positive and SNCG negative cells (data not shown).

Metastasis in orthotopic nude mice model. Since SNCG was highly expressed in the infiltrating breast cancer cells relative to benign or non-invasive *in situ* carcinomas (2), we are interested in studying whether SNCG is an instigator of metastasis or merely a correlative product during breast cancer progression. The effect of SNCG expression on metastasis was assayed in an *in vivo* orthotopic (mammary fat pad) nude mouse model. Two independent experiments were done to confirm reproducibility, and the data from these experiments are summarized in Table 1. After a lag phase of 10 days, mice given implants of both SNCG positive and SNCG negative cells developed tumors. There was no difference in tumor incidence between neo-435 and SNCG-435 clones. Starting at about 20 days after inoculation, tumor necrosis was observed in tumors derived from SNCG-435-1 and SNCG-435-3 cells. Neo-435-1 and neo-435-2 also developed some tumor necrosis but with less intensity. Consistent with the *in vitro* similar growth rates, at the end of 40 days after injection, there was no significant difference in primary tumor size between neo-435 and SNCG-435 clones.

To study tumor dissemination, axillary lymph nodes and lungs were first examined physically at autopsy and then subjected to microscopic examination for morphologic evidence of tumor cells by light microscopy on H & E stained paraffin sections. For the axillary lymph node, the average weight was 15 mg for neo-435 mice, and was increased to 44 mg for SNCG-435 mice. The increased lymph node weight reflects the invaded breast tumors. A representative H & E stained sections for neo-435 and SNCG-435 lymph nodes are presented (Fig. 2). Microscopic examination indicated that SNCG-435-1 and SNCG-435-3 mice showed an average significantly higher lymph node positivity of 64% and 77% as compared to the average of 27% from SNCG negative neo-435-1 and neo-435-2 mice (Table 1). For the lung metastasis, the numbers of visible nodules on the surface of the lungs were dramatically increased from an average of 1 for neo-435 mice to the average of 23 for SNCG-435 mice (Table 1). The representative lungs were shown in Fig. 3. When these lungs were examined microscopically, large numbers of micro-metastasis were observed in SNCG-435 mice; the lungs from neo-435 mice had significantly less micro-metastasis (data not shown). A representative H & E stained sections for neo-435 and SNCG-435 lungs are presented (Fig. 4). To our knowledge, human breast cancer cells usually do not form such profound regional and metastatic tumor dissemination (visible lung nodules) in the spontaneous mammary fat pad nude mouse model. This dramatic SNCG-stimulated metastasis suggests a role of SNCG as one of key positive regulator for breast cancer invasion and metastasis. The overexpression of SNCG in malignant infiltrating breast epithelial cells compared to the low level expression in the non-invasive in situ carcinoma (2) suggests that SNCG expression is a meaningful marker for breast cancer malignant progression and may signal the more advanced invasive/metastatic phenotype of human breast cancer. In this regard, the up-regulation of SNCG expression may facilitate the breast cancer progression leading to metastasis.

Matrix metalloproteinase (MMP) activity. In an effort to investigate the molecular mechanisms underlying SNCG-induced metastasis, we studied several invasion-related factors including MMP and cell motility. The amyloid protein has been recently demonstrated to be a strong stimulator for MMP-2 and MMP-9 expression in astrocytes (12). It is well established that the overproduction and unrestrained activity of MMPs and particular MMP-2 and MMP-9 has been linked to malignant conversion of a variety of different tumor cells (13-20) including mammary tumors (16-20). It is interesting to test whether SNCG, an amyloid-related protein, stimulates MMP-2 and MMP-9 expression in breast cancer cells and leads to the more metastatic phenotype. We investigated if the SNCG overexpression would increase the MMP activity of MDA-MB-435 cells. In this regard, the pooled conditioned media (CM) from two SNCG negative cells and the pooled CM from two SNCG positive cells were concentrated and subjected to MMP enzymatic assay. As shown in Fig. 5, no significant differences of basal levels proteolytic activities were observed between neo-435 and SNCG-435 clones. Mammalian MMPs are usually secreted as latent proenzymes (zymogen) and require activation for their enzymatic activity. Incubation of the CMs with MMP activator organomercurial compounds APMA resulted in approximately 2-fold increase in proteolytic activity for CMs from both neo-435 and SNCG-435 clones. However, no significant difference on APMA-activated MMP activities was observed between neo-435 and SNCG-435 clones. Since the measured enzymatic activity represents the net MMP activity reflecting the balance between activated MMPs and the tissue inhibitors of metalloproteinase (TIMPs), our data suggest that SNCG-induced metastasis may not be mediated by regulation of MMP and TIMP.

Stimulation of invasiveness and motility of MDA-MB-435 cells by SNCG. We used an *in vitro* reconstituted basement membrane (Matrigel) invasion assay to determine the effect of SNCG on cell invasion. All three SNCG negative cells parental MDA-MB-435, neo-435-1, and neo-435-2 cells were moderately invasive. At the end of 48-h incubation, approximately average 250 of SNCG negative cells had crossed the Matrigel barrier. A significant stimulation in invasiveness was noted in two SNCG positive clones with a 3-fold increase for SNCG-435-1 cells and a 4.3-fold increase for SNCG-435-3 cells (Fig. 6A). We also investigated the effect of SNCG on cell migration (without Matrigel). A similar SNCG-stimulated pattern on migration was observed. At the end of 24-h incubation, SNCG-435-1 cells migrated 4-fold and SNCG-435-3 cells migrated 4.2-fold over that of average SNCG negative cells (Fig. 6B). The similar magnitude of the invasion-stimulating and migration-stimulating activity of SNCG suggests the increased invasion in SNCG clones may be mediated by alternation of cell motility. In order to determine whether the increased cell motility is mediated by chemotaxis due to the different concentrations of serum or chemoattractants in the top and bottom chambers, we picked up SNCG-435-3 and neo-435-1 cells and compared their migration at three different culture conditions: serum free, serum with gradient, and serum without gradient. As shown in Fig. 7, although the migration was relative low in the serum free conditions, there was a 2.8-fold increase in migration in SNCG-435-3 cells compared with that in neo-435-1 cells. When 2% serum was added in the top chamber, the migration of both SNCG positive and negative cells increased significantly. However, the migration of SNCG-435-3 cells was not affected by serum gradient. Approximately 1,600 SNCG-435-3 cells migrated into the bottom chamber contained either same 2% serum or 10% serum. These data suggest that the increased migration in SNCG positive cells is not likely to be mediated by chemotaxis but rather by cellular intrinsic high motile features.

Many breast tumors go through a series of events from the time of initial detection to the formation of the lethal invasive and metastatic stage. The ability of the tumor cells to cross the basement membrane of epithelial barriers it encounters during metastasis is believed to be a dominant factor in its invasive potential. According to the three-step hypothesis of invasion (21), cell adhesion, local proteolysis and subsequent migration or motility are key steps to traversal of basement membrane and connective tissue. In the present report, we provided evidences linking overexpression of a neurotic protein SNCG, a previously identified breast cancer specific gene (2), in human breast cancer cells with increased motility and invasive activity *in vitro* and a profound augmentation of metastasis *in vivo*.

Synuclein proteins share structural resemblance to apolipoproteins, but are abundant in the neuronal cytosol and present in enriched amounts at presynaptic terminals (9). Synucleins have been specifically implicated in two diseases: AD and PD. In AD patients, a peptide derived from SNCA forms an intrinsic component of plaque amyloid (9). In PD patients, an SNCA allele is genetically linked to several independent familial cases, and the protein appears to accumulate in Lewy bodies (9). We currently do not know the significance of the involvement of a neurotic protein SNCG in cancer metastasis in general. Recently, SNCA and SNCB were identified as two abundant proteins through their reactivity on immunoblots with a monoclonal antibody recognizing microtubule-associated protein (MAP) tau (6). In eukaryotic cells, microtubules, actin, and intermediate filaments interact to form the cytoskeletal network involved in determination of cell architecture, mitosis, differentiation and motility (22). Cytoskeletal organization and dynamics depend on protein self-associations and interactions with regulatory elements such as MAPs. There is increasing evidence that microtubule-associated proteins

including tau play a critical role in inducing microtubule assembly and in controlling the dynamic instability of microtubules, thus controlling the state of their assembly and organization in cells (reviewed in ref. 22). SNCG may interact with MAPs, regulate the cytoskeletal organization and dynamics, and thus lead to increased motility. Nevertheless, our data indicate that the increased expression of SNCG correlates with breast progression (2) and leads to more malignant metastatic phenotype. We also demonstrated that SNCG expression in breast cancer cells was subjected to cytokine regulation and was dramatically suppressed by tumor growth inhibitor oncostatin M (OM) and this OM-induced transcriptional suppression of SNCG gene was associated with OM-mediated growth inhibition (23). OM is an anti-tumor cytokine mainly produced by activated T cells and macrophages (24) and its growth suppressing activity was well studied in breast cancer cells (25-27). One characteristic of the host response to tumor progression is the infiltration of tumors by macrophages and T lymphocytes. Production of tumor suppressing cytokines in a timely and locally (in situ) released fashion may represent an important function of the host defense system to suppress tumor progression. From this prospective view, the dramatic suppression of SNCG expression in malignant breast cells by OM may represent the host-mediated tumor suppression leading to the inhibition of breast cancer progression.

This is the first report indicating the potential involvement of synuclein in the non-neurotic disease. An elucidation of the reasons for SNCG overexpression in infiltrating breast cancer and SNCG-induced metastasis may shed some light on the pathogenesis of not only breast cancer progression but also neurodegenerative disorders.

III-3. Transcriptional suppression of the breast cancer-specific synuclein γ (*SNCG*) expression by the growth inhibitory cytokine oncostatin M (see attached manuscript 2)

Time- and concentration-dependence of OM-mediated suppression of *SNCG* mRNA expression in breast cancer cells. Breast cancer cell lines MCF-7, ZR-75-1, and H3922 were shown growth-inhibited by OM (28-29). Because *SNCG* mRNA is not expressed in MCF-7 cells, and is expressed at very low level in ZR-75-1 cells, but expressed at a relatively high level in H3922 cells (2), we examined the effect of OM on *SNCG* mRNA expression in H3922 cells. The results in Figure 1 demonstrated a marked time-dependent suppression of *SNCG* mRNA level by OM. Treatment of H3922 cells with OM initiated an immediate decrease of *SNCG* mRNA as early as 30 minutes. By 4h treatment, the level of *SNCG* mRNA was decreased to 70% of that in control cells and by 24 h the mRNA was completely undetectable. The suppressive effect of OM is persistent, as *SNCG* mRNA was not yet detected in H3922 cells after culture the cells for two days in OM-free medium, *SNCG* mRNA was partially expressed after three days of withdrawing OM from the medium (data not shown).

OM-suppressed *SNCG* transcription was also concentration dependent. After 6h treatment, OM at concentrations as low as 0.2ng/ml caused significant decrease of *SNCG* expression (58% of control), whereas maximal suppressions were observed at concentrations of 1-5ng/ml (Figure 2). This concentration dependence is comparable to those for inhibition of cell growth (28-29). These data suggest that inhibition of cell growth and suppression of *SNCG* transcription is correlated.

Transcriptional regulation of *SNCG* expression by oncostatin M. To determine whether the down regulation of *SNCG* expression by OM occurs at the transcriptional or post-

transcriptional level, we conducted nuclear run-on assays to measure the relative transcription rate of *SNCG* in control cells and in the cells treated with OM. As shown in Figure 3, Treatment of H3922 cells with OM for 16 h, decreased the level of actively transcribed *SNCG* mRNA to 28.5% of that in untreated cells. Data were normalized by the signals observed in the *GAPDH* slots. The level of reduction of *SNCG* transcripts is consistent with the results obtained from northern blot analysis, suggesting a transcriptional regulation mechanism. To confirm this observation, *SNCG* mRNA stability was examined. Control cells and the cells treated with OM for 6 h were exposed to actinomycin D. Total RNAs isolated from the cells at various actinomycin D exposure time points were subjected to northern blot analyses of *SNCG*. (Figure 4). Although OM treatment reduced the level of *SNCG* mRNA to approximately 50% of that in control cells, the *SNCG* mRNA levels in both the control cells and the OM-treated cells were not decreased by actinomycin D. In contrast, the *c-Myc* mRNA levels were rapidly reduced by treatment with actinomycin D. These data suggest that the *SNCG* mRNA stability was not altered by OM, and that the *SNCG* mRNA is relatively stable. An attempt to treat cells with actinomycin D for a longer period of time was not successful due to the actinomycin D-mediated toxicity in H3922 cells. These results together with the data generated from the nuclear run-on assay suggest that *SNCG* gene expression was suppressed by OM mainly at the transcriptional level.

Over expression of *SNCG* gene in breast cancer cells stimulates cell growth. To further investigate the correlation between *SNCG* expression and proliferation of breast cancer cells, we selected MCF-7 cells as a recipient cell line for *SNCG* mediated gene transfection due to its lack of endogenous *SNCG* mRNA expression (2). MCF-7 cells were transfected with a plasmid vector containing a neomycin resistance gene (neo-MCF clones), or the same vector containing a full-length *SNCG* cDNA (*SNCG*-MCF clones). Individual clones were initially screened by *in situ* hybridization on slides with a specific *SNCG* antisense probe, and the positive clones were subjected to northern blot analysis. Fig. 5 shows the northern blot analysis of *SNCG* expression in selected clones. All selected *SNCG*-MCF clones expressed *SNCG* mRNA transcripts. In contrast, none of the neo-MCF clones produced any detectable *SNCG* mRNA. No changes in morphology were observed in these clones.

To determine whether *SNCG* over expression affects the growth of the transfected cells, the growth rates of *SNCG* positive MCF-7 clones (*SNCG*-MCF-2 and *SNCG*-MCF-6) were compared to that of *SNCG* negative MCF-7 cells (neo-MCF-1 and neo-MCF-2) in a monolayer culture. As shown in Figure 6, the cell growth was significantly stimulated in *SNCG* transfected cells compared to muck-transfected cells to approximately 3.2-fold ($p < 0.001$ by Student's *t-test*).

OM-specific receptor type II mediates down regulation of *SNCG* gene expression. The biological activities of OM can be mediated through two types of receptor complexes, the LIF/OM shared receptor (type I) and OSM-specific receptor (OSMR, type II) (30-31). Previous studies conducted in our laboratory showed that the growth-inhibitory activity of OM in the H3922 breast cancer cells is mediated through OM-specific receptor type II (28,32). Although the type I OM receptor that mediates the actions of LIF and OM is also expressed in these cells, LIF did not inhibit the growth of these cells, instead slightly stimulated their growth (28). In addition, previous study showed that IL-6 and IL-11 do not affect the growth of these cells either (28). To determine whether the effect of OM on *SNCG* gene expression is mediated through the type II OM-specific receptor, the effect of LIF, along with IL-6 and IL-11 on *SNCG* expression

was compared with OM. H3922 cells were treated with individual cytokines for 24h at a concentration of 100ng/ml. The results of northern blot analysis show that *SNCG* mRNA expression was suppressed by OM, but not suppressed by LIF, or IL-6 and IL-11 (Figure 7). These data suggest that the type II OM specific-receptor transmit OM elicited signals that lead to the repression of *SNCG* transcription.

IV. CONCLUSIONS

The process of metastasis in breast cancer accounts for the inability to cure all but the earliest stages of the disease. Metastasis is proposed to depend on five major activities: angiogenesis, cellular attachment, proteolysis, and migration through the barrier into the secondary sites, and, of course, colonization and proliferation in the distant organs. We have recently identified and cloned a putative breast cancer specific gene, SNCG, which was **(a)** highly expressed in mammary gland relative to other organs and was **(b)** high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library. Preliminary studies demonstrated that: 1) SNCG expression was a stage-specific in human breast: undetectable in normal or benign breast lesions, low level and partial expression in low grade ductal carcinoma *in situ* but extremely high level in advanced infiltrating breast cancer; 2) SNCG expression in human breast cancer cells is dramatically suppressed by tumor growth inhibitor oncostatin M (OM), a cytokine predominantly produced by activated T cells and macrophages; 3) overexpression of SNCG in breast cancer cells led to a significant increase in cell motility and invasiveness *in vitro* and a profound augmentation of metastasis *in vivo*. The use of SNCG gene could be of importance in differentiating atypical proliferative breast lesions or noninvasive carcinoma *in situ* from malignant and invasive cancer and may be useful in screening of breast biopsies for potential abnormalities. In addition, if overexpression provides a therapeutic target, then SNCG may be useful in clinical management and treatment of breast cancer.

V. PUBLICATIONS

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VI. LIST OF PERSONNEL RECEIVING PAY FROM THIS EFFORT

None.

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Identification of a Breast Cancer-specific Gene, *BCSG1*, by Direct Differential cDNA Sequencing[1]

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ABSTRACT

A high-throughput direct-differential cDNA sequencing approach was employed to identify genes differentially expressed in normal breast as compared with breast cancer. Approximately 6000 expressed sequence tags (ESTs) from cDNA libraries of normal breast and breast carcinoma were selected randomly and subjected to EST-sequencing analysis. The relative expression levels of more than 2000 unique EST groups were quantitatively compared in normal *versus* cancerous breast. Of many putative differentially expressed genes, a breast cancer-specific gene, *BCSG1*, which was expressed in high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified as a putative breast cancer marker. *In situ* hybridization analysis demonstrated stage-specific BCSG1 expression as follows: BCSG1 was undetectable in normal or benign breast lesions, showed partial expression in ductal carcinoma *in situ*, but was expressed at an extremely high level in advanced infiltrating breast cancer. The predicted amino acid sequence of *BCSG1* gene has a significant sequence homology to the non-amyloid β protein fragment of the Alzheimer's disease amyloid protein. BCSG1 overexpression may indicate breast cancer malignant progression from benign breast or *in situ* carcinoma to the highly infiltrating carcinoma.

INTRODUCTION

The onset and progression of breast cancer is accompanied by multiple genetic changes that result in qualitative and quantitative alterations in individual gene expression (1). Our hypothesis is that many of these quantitative genetic changes manifest themselves as alterations in the cellular complement of novel transcribed mRNAs. Identification of these mRNAs, if sufficiently characterized, could provide clinically useful information for patient management and prognosis while enhancing our understanding of breast cancer pathogenesis. Although pathological end points such as tumor size, lymph node status, and status of estrogen receptor and progesterone receptor remain the most useful guides in prognosis and in selecting treatment strategies for breast cancer (2), there is a need to further investigate the molecular mechanisms that determine the properties of an individual tumor, *e.g.*, probability of metastasis. Although numerous prognostic factors have now been identified, few have contributed to defining the clinical response to therapy.

Identification of quantitative changes in gene expression that occur in the malignant mammary gland, if sufficiently characterized, may yield novel molecular markers that may be useful in the diagnosis and treatment of human breast cancer. Several differential cloning methods, such as differential display PCR and subtractive hybridization, have been used to identify the genes differentially expressed in breast cancer biopsies, as compared to normal breast tissue controls (3–7). However, these investigations have involved the relatively time- and labor-intensive steps of subcloning, library screening, and cDNA sequencing of individual genes (4, 8). On the other hand, creation of libraries is a rapid method used to identify or "tag" sequences that are expressed in specific tissues (9, 10). Since the introduction of the EST[3] sequencing approach, many novel human genes have been discovered (9, 10). The advantage of this methodology, compared to isolation and sequencing of individual cDNAs, is that a large number of sequences can be "catalogued" with small amounts of sequencing data.

With the availability of tens of thousands of ESTs, researchers now shift their attention to the unveiling of the expression profile of individual genes or patterns of genes in normal *versus* diseased states. Several newly developed strategies, such as the serial analysis of gene expression (11) and cDNA microarray (12) methods, have demonstrated potential for broad application for quantitative analysis of differential patterns of gene expression. Within this context, we undertook a search, using the differential cDNA sequencing approach, for isolation of differentially expressed ESTs and the possible presence of the new marker genes for breast cancer. In this initial report, we describe a novel BCSG named *BCSG1* that is overexpressed in advanced infiltrating breast cancer cells but not in normal or benign breast lesion. The expression pattern of BCSG1 may be a meaningful marker in the development of breast cancer.

MATERIALS AND METHODS

Reagents. Restriction enzymes, T7 polymerase, random primer DNA labeling kit, and digoxigenin-labeled nucleotides were obtained from Boehringer Mannheim (Indianapolis, IN). [^{32}P]dATP was purchased from Amersham Corp.

Differential cDNA Sequencing. We have used EST analysis to search for new genes differentially expressed in breast cancer *versus* normal breast tissue. A data base containing approximately 500,000 human partial cDNA sequences (ESTs) has been established in a collaborative effort between the Institute for Genomic Research and Human Genome Sciences, Inc., using high-throughput automated DNA sequence analysis of randomly selected human cDNA clones (10). RNAs from a stage III breast carcinoma and patient-matched normal breast were isolated and subjected to preparation of cDNA libraries. EST-automated DNA sequence analysis was performed on randomly selected cDNA clones. Both libraries had about 60% novel gene sequences, which did not match exactly to published human genes. A total of 3048 ESTs from breast cancer cDNA library and 2886 ESTs from the normal breast cDNA library were randomly picked and sequence analyzed. The ESTs with overlapping sequences were grouped into unique EST groups, with each EST group representing a gene or a family of sequence-related genes. Each unique EST group without overlapping sequences was analyzed for its relative expression by examining the number of expressed individual ESTs in the libraries of normal *versus* diseased tissues. There were more than 2200 EST groups that were analyzed for quantitative comparison of EST "hits" in the pair of cDNA

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[3] The abbreviations used are: EST, expressed sequence tag; BCSG, breast cancer-specific gene; Aβ, amyloid β protein; AD, Alzheimer's disease; DCIS, ductal carcinoma *in situ*.

Table 1 *Partial list of differentially expressed genes in normal versus cancerous breasts identified by differential cDNA sequencing*

Complementary DNA libraries were established from a stage III breast carcinoma and patient-matched normal breast. A total of 5934 ESTs were randomly picked and sequence analyzed. More than 2200 distinctive EST groups were analyzed for quantitative comparison of EST hits in the pair of cDNA libraries from breast cancer *versus* normal breast as described in "Materials and Methods." The same EST groups were also analyzed by examining the tissue-specific expression against the total of 500.000 ESTs from a variety of different cDNA libraries. Only a unique EST group with more than three breast-specific EST hits was listed, and the rest of the several dozen EST groups with fewer than four breast-specific EST hits were omitted in this list.

Genes more abundant in breast cancer

	ESTs	
Class I Genes	Cancer	Normal
Breast basic conserved gene	33	9
Cathepsin D	5	1
M_r 67,000 laminin receptor	4	0
Elongation factor 1	13	5

Genes more abundant in normal breast

	ESTs	
Class II Genes	Cancer	Normal
Matrix Gla protein	0	8
M_r 23,000 highly basic protein	3	11

Genes as breast-specific and differentially expressed

	ESTs		
Class III Genes	NB[a]	BC[b]	All tissues
BCSG1	1	6	8[c]
BCSG2	0	7	7
BCSG3	0	5	5
BCSG4	4	0	4
BCSG5	0	4	4

[a] normal breast; [b] breast cancer; [c] seven ESTs from breast libraries and one EST from brain library.

libraries from normal breast *versus* breast cancer by examining the expression of individual EST sequences. The number of EST hits in the libraries reflects the relative expression or mRNA transcript copy numbers of the EST. This direct differential cDNA sequence, utilizing the direct EST sequencing analysis simultaneously on a pair of cDNA libraries made from normal breast and breast cancer tissue, was used to study the expression profile of individual genes and patterns of genes in normal breast *versus* breast cancer tissue.

Tissue-specific Expression Analysis. Analysis of relative expression of breast-derived ESTs *versus* their expression in other tissues was performed. The differentially expressed EST groups identified by differential cDNA sequence were analyzed for tissue-specific expression against the total of 500,000 ESTs from a variety of different cDNA libraries.

Northern Analysis. Total RNA was extracted from tissues according to the method of Chomczynski and Sacchi (13). The RNA from human breast cancer cells was prepared using the RNA isolation kit RNAzol B (Tel-Test, Inc.) based on the manufacturer's instruction. Equal aliquots of RNA were electrophoresed in a 1.2% agarose gel containing formaldehyde and transferred to nylon membrane (Boehringer Mannheim). The membrane was prehybridized with ExpressHyb hybridization solution (Clontech, Inc.) at 68°C for 30 min. The hybridization was carried out in the same solution with ^{32}P-labeled BCSG1 probe (1.5×10^6 cpm/ml) for 1 h at 68°C. The membrane was then rinsed in 2× SSC containing 0.05% SDS three times for 30 min at room temperature, followed by two washes with 0.1× SSC containing 0.1% SDS for 40 min at 50°C. The full-length BCSG1 cDNA was isolated from the Bluescript vector following *Eco*RI and *Xho*I digestion and was used as a template for preparation of a random-labeled cDNA probe.

***In Situ* Hybridization.** *In situ* hybridization was carried out as described (14). Briefly, deparaffinized and acid-treated sections (5 μm thick) were treated with proteinase K, prehybridized, and hybridized overnight with digoxigenin-labeled antisense transcripts from a BCSG1 cDNA insert. The BCSG1 antisense probe is a 550-bp full-length fragment. The probe was generated by a *Pst*I cut of BCSG1 cDNA plasmid and followed by T7 polymerase. Hybridization was followed by RNase treatment and three stringent washings. Sections were incubated with mouse antidigoxigenin antibodies (Boehringer Mannheim) followed by the incubation with biotin-conjugated secondary rabbit antimouse antibodies (DAKO). The colorimetric detections were performed with a standard indirect streptavidin-biotin immunoreaction method using the Universal LSAB Kit (DAKO) according to the manufacturer's instructions.

RESULTS

Molecular Cloning of BCSG1 cDNA. We generated cDNA libraries from breast cancer biopsy specimens and patient-matched normal breast and analyzed these libraries by EST sequencing. Approximately 6000 ESTs were analyzed and assigned to different groups based on sequence overlapping, and 2200 unique EST groups were first analyzed for relative expression in the cDNA libraries from normal breast *versus* breast cancer tissue and then subjected to tissue-specific expression by examining the tissue origins of individual EST sequences against a large population of ESTs derived from a variety of different tissue types. As a result, we identified three classes of EST groups that were differentially expressed in normal breast *versus* breast cancer tissue. As a demonstration of this approach, Table 1 shows a partial list of three classes of genes that are differentially expressed in normal breast *versus* breast cancer tissue. Class I represents the genes more abundant in breast cancer than in normal breast and includes cathepsin D, a well-studied steroid regulated extracellular matrix-degrading proteinase (15–17). Cathepsin D is thought to play a role in breast cancer metastasis (15–17) and has been proposed as a prognostic marker in breast cancer progression (18–21). As listed, there were five cathepsin D ESTs sequenced in the breast cancer cDNA library and only one EST in the normal breast cDNA library. Another proposed breast cancer metastasis-related gene and a prognostic marker for breast cancer, M_r 67,000 laminin receptor (22–26), was also picked up in this class by the differential cDNA sequencing approach. Class II represents genes that are more abundant in normal breast than in breast cancer.

```
1    M D V F K K G F S I A K K G V V G A V E    BCSG1
1    M D V F M K G L S K A K E G V V A A A E    *Human AD Amyloid

21   K T K Q G V T E A A E K T K E G V M Y V    BCSG1
21   K T K Q G V A E A A G K T K E G V L Y V    *Human AD Amyloid

41   S A K T K E N V V Q S V T S V A E K T K    BCSG1
41   S S K T K E G V V H G V A T V A E K T K    *Human AD Amyloid

61   E Q A N A V S K A V V S S V N T V A T K    BCSG1
61   E Q V T N V G G A V V T G V T A V A Q K    *Human AD Amyloid

81   T V E E A E N I A V T S G V V R K E D L    BCSG1
81   T V E G A G S I A A A T G F V K K D Q L    *Human AD Amyloid

101  K P S A P Q Q E G E A S K E K E E V A E    BCSG1
101  G H N E E G A P Q E G I L E D M P V D P    *Human AD Amyloid

121  E A Q S G G D                             BCSG1
121  D N E A Y E M P S E E G Y Q D Y E P E A    *Human AD Amyloid
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*Non-Aß component of Alzheimer's disease (AD) Amyloid

Fig. 1. Comparison of the predicted amino acid sequence with the sequence of non-Aβ component of AD amyloid protein using SwissProt. After optimal alignment using the clustal method of the MegAlign Program from the DNASTAR software package, the putative protein shows a 54% sequence identity with the non-Aβ fragment of human AD amyloid protein. Conserved amino acids are underlined.

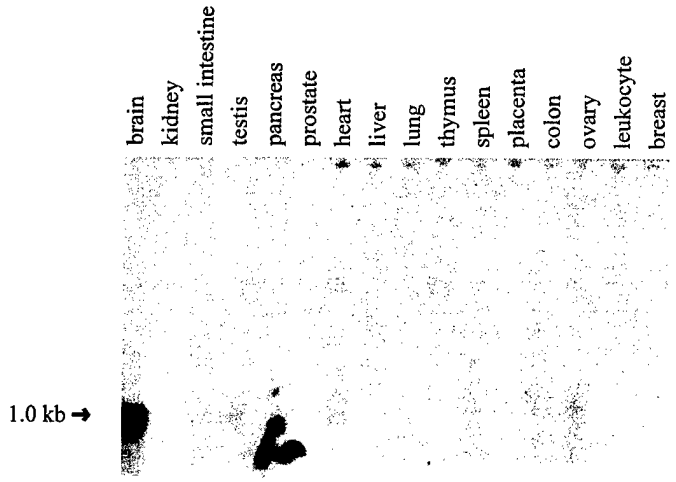

Fig. 2. The expression of *BCSG1* gene in a variety of normal human adult tissues. Twenty μg of total RNA from each of the above tissues were analyzed in Northern blot using a random primer probe. A strong hybridizing band of about 1 kb was recognized in the lane corresponding to RNA from adult brain. A weak 1-kb transcript was also detected in testis, heart, spleen, colon, and ovary.

Although the genes in classes I and II are differentially expressed in normal breast *versus* breast cancer tissue, these genes are not unique to breast tissues. Class III is a special group of genes that are selectively expressed in breast relative to other tissue types. The tissue-specific expression of the unique gene was searched against approximately 500,000 ESTs using the BLAST program (27). None of these BCSGs except the first one matched with any sequences in public gene sequence databases. The automated screening revealed a group of eight ESTs encoding a novel *BCSG1* gene from the partial cDNA database containing approximately 500,000 ESTs. Of the eight distinctive EST clones in BCSG1, seven of them were discovered in breast cDNA libraries and only one in a brain library. Of the seven EST clones discovered in the breast cDNA libraries, six of them were identified in the breast tumor library and only one in the normal breast library. BCSG1 was chosen for analysis as a first putative breast cancer marker gene because (*a*) its sequence has been matched with the sequence in the public gene sequence database; and (*b*) most of the individual EST sequences in BCSG1 were derived from a breast tumor cDNA library. After sequencing analysis of all six EST clones derived from the breast cancer library, one EST clone was found to have a complete full-length sequence. The open reading frame of the resulting full-length gene is predicted to encode a 127-amino acid polypeptide. Comparison of the predicted amino acid sequence with the sequence of a similar human protein is shown in Fig. 1. After optimal alignment, the putative BCSG1-encoded protein shows 54% sequence identity with the recently cloned non-Aβ fragment of human AD amyloid protein (28).

Tissue Expression. The expression of *BCSG1* gene in a variety of normal human tissues were analyzed by Northern blotting (Fig. 2). As expected, the Northern blot showed that BCSG1 was abundantly expressed as a 1-kb transcript in brain, which is the rich source for the *AD* amyloid family gene. Similar bands with much lower accumulations in their relative intensities were also obtained in ovary, testis, colon, and heart. By contrast, none of them was present in other specimens analyzed, such as breast, kidney, liver, prostate, lung, small intestine, thymus, and placenta.

Expression of BCSG1 in Human Breast Cancer Cells. In an attempt to evaluate the potential biological significance of BCSG1 on human breast cancer development and progression, we studied *BCSG1* gene expression in human breast cancer cells. Northern blot (Fig. 3) detected the 1-kb BCSG1 transcript in two of four lines derived from pleural effusion and four of four lines detected from ductal infiltrating carcinomas. Among these lines, H3922 expressed the highest level of BCSG1 mRNA. The absence of BCSG1 mRNA in some breast cancer cell lines may suggest that the expression of *BCSG1* gene requires specific *in vivo* conditions, or that it is induced by interactions between the tumor cells and stromal cells.

To localize the cellular source of the BCSG1 expression and to further assess the biological relevance of the overexpression of BCSG1 in breast cancers, we next performed *in situ* hybridization on fixed breast sections from 20 infiltrating carcinomas, 15 DCISs, and 18 benign breast lesions, including 5 reduction mammoplasty specimens, 8 breast hyperplasias, and 5 fibroadenomas. In these experiments, we examined two aspects of BCSG1 expression, including the tissue localization (stromal *versus* epithelial) and the correlation of BCSG1 expression and breast cancer malignant phenotype. There was a wide variation in staining intensity for BCSG1 expression among the human breast cancer specimens. Because the colorimetric *in situ* hybridization is not quantitative, the tissue samples were classified into either positive or negative staining for BCSG1 expression; no attempt was made to differentiate the levels of expression of BCSG1 among positive-staining specimens. The negative cases were confirmed with at least two independent experiments. All stainings were reviewed by at least two people. Fig. 4 shows a representative *in situ* hybridization for BCSG1. We found a strongly positive BCSG1 hybridization in neoplastic epithelial cells of highly infiltrating breast carcinomas (Fig. 4, *A* and *B*). The expression of BCSG1 mRNA was detectable in the neoplastic epithelial cells in 17 of 20 infiltrating breast carcinomas. No expression of BCSG1 was detected in the stromal cells. In contrast, expression of BCSG1 was absent in 16 out of 18 cases of normal or benign breast lesions. A representative negative staining of BCSG1 in normal ductal breast epithelial cells (Fig. 4*E*), a benign proliferative breast lesion (Fig. 4*F*), and a benign fibroadenoma (Fig. 4*G*) are presented. Furthermore, as demonstrated in Fig. 4*B* for a highly invasive breast carcinoma, no detectable signal of BCSG1 expression was evident in the residual normal lobular breast epithelial cells, although the surrounding invasive breast carcinoma cells were stained positive for BCSG1 expression. The presence of BCSG1 transcript in human breast tissue and its overexpression in breast carcinomas are consistent with our differential cDNA sequencing cloning strategy, which suggests a possible

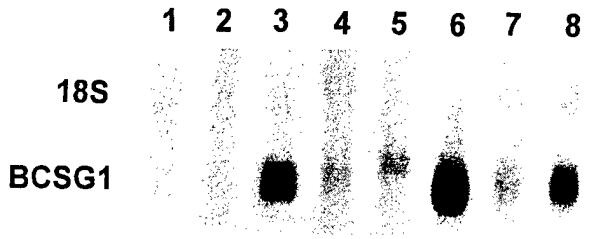

Fig. 3. Northern blot analysis of BCSG1 expression in human breast cancer cell lines. Total RNA was isolated and analyzed (20 μg/*Lane*) by Northern blot. After hybridization and washing, the filter was exposed to X-ray film for 48 h. The integrity and the loading control of the RNAs were ascertained by direct visualization of the 18 S rRNA in stained gel. *Lane 1*, H3396 (derived from pleural effusion); *Lane 2*, MCF7 (derived from pleural effusion); *Lane 3*, SKBR-3 (derived from pleural effusion); *Lane 4*, MDA-MB-231 (derived from pleural effusion); *Lane 5*, H3914 (derived from infiltrating ductal carcinoma); *Lane 6*, H3922 (derived from infiltrating ductal carcinoma); *Lane 7*, ZR-75-1 (derived from infiltrating ductal carcinoma); *Lane 8*, T47D (derived from infiltrating ductal carcinoma). Cell lines T47D, ZR-75-1, SKBR-3, MCF-7, and MDA-MB-231 are from American Type Culture Collection; all other lines were isolated initially at Bristol-Myers Squibb Pharmaceutical Research Institute.[4]

[4] J. Liu, M. J. Spence, P. M. Wallace, K. Forcier, I. Hellstrom, and R. T. Vestal. Oncostatin M-specific receptor mediates inhibition of breast cancer cell growth, antagonism of growth factors, and down regulation of c-*myc* proto-oncogene, submitted for publication.

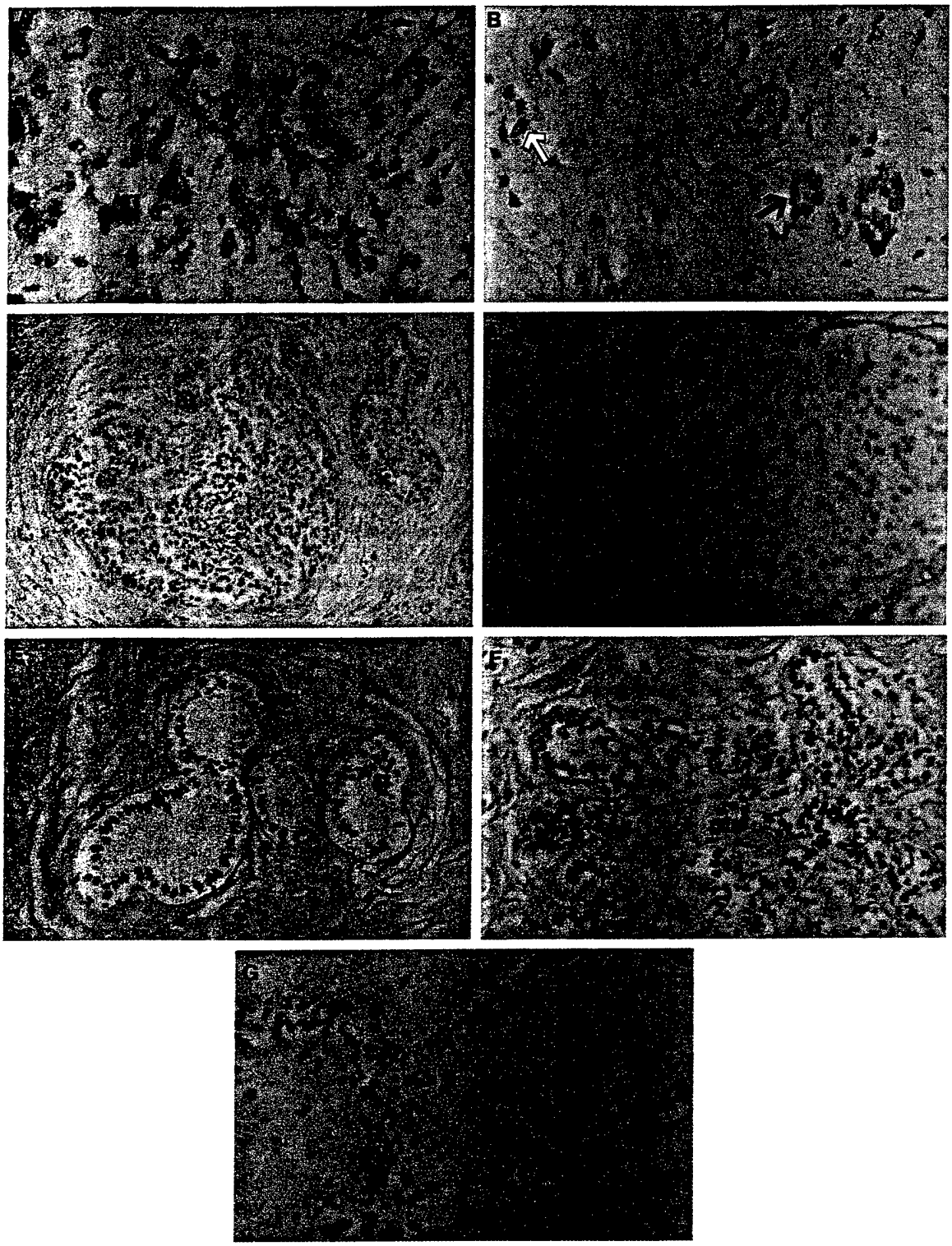

Fig. 4. *In situ* hybridization analysis of BCSG1 expression in human breast. Cells labeled with *brown* indicate *BCSG1* gene expression. All sections were counterstained lightly with hematoxylin for viewing negatively stained cells. *A*, a highly infiltrating breast carcinoma showed a very strong BCSG1 expression in virtually every malignant cell. *B*, high-magnification view of breast cancer cell invasion to normal lobule. *Solid arrow*, negatively stained residual normal lobular epithelial cells; *open arrow*, positively stained invasive cancer cells. *C*, Comedo-type DCIS showing BCSG1 staining. *D*, negative staining of BCSG1 in a non-Comedo-type DCIS. *E*, negative staining of normal ductal epithelial cells. *F*, negative staining of epithelial cells in a benign hyperplasia. *G*, negative staining of a benign fibroadenoma.

role or a biomarker of up-regulation of BCSG1 in the development of breast cancer.

It is interesting to note that although a strong BCSG1 signal was easily detected in the malignant breast epithelial cells of infiltrating breast carcinoma, the *in situ* carcinomas showed different BCSG1 expression patterns. Among 15 DCISs (8 are Comedo type and 7 are non-Comedo type), 8 specimens stained negatively (Fig. 4*D*) and 7 specimens were positive (Fig. 4*C*). Interestingly, six of seven BCSG1-positive DCIS samples were Comedo-type DCIS, and only one was non-Comedo type; among the BCSG1-negative specimens, there were six non-Comedo-type DCISs and only two Comedo-type DCISs. These results, which demonstrated a stage-specific BCSG1 expression

from virtually no detectable expression in normal or benign breast to partial expression (7 of 15) in the *in situ* breast carcinoma and to the high expression (17 of 20) in the infiltrating malignant breast carcinomas, suggest an association of BCSG1 expression with breast cancer malignant progression. On the basis of this BCSG1 expression pattern, we propose that BCSG1 may be used potentially as a breast cancer progression marker.

DISCUSSION

More than 190,000 new cases of breast cancer are diagnosed in the United States every year, with incidence increasing by approximately 1% annually (29, 30). Studies linked to the discovery of new genetic markers will provide new information leading to the understanding of breast cancer development and progression. There are two classes of genes affecting tumor development. Genes influencing the cancer phenotype that act directly as a result of changes (*e.g.*, mutation) at the DNA level, such as *BRCA1*, *BRCA2*, and *p53*, are called Class I genes. The Class II genes affect the phenotype by modulation at the expression level. Development of breast cancer and subsequent malignant progression is associated with alterations of a variety of genes of both classes. Many new predictive and prognostic factors have been proposed and studied for breast cancer. HER 2/neu-positive tumors respond poorly to endocrine treatment (31, 32). p53 alteration has an indication of poorer prognosis and poor response to tamoxifen (33, 34). The lack of Nm23 expression has an indicative value of metastatic potential and poor prognosis in invasive ductal carcinoma (35). Cathepsin D, a protease suggested to have a role in breast cancer, appears to affect the potential for invasive growth (11, 14, 36). Positive immunostaining of tumor sections with Factor VIII antibodies seems to be a marker for angiogenesis (37–39). It has been postulated that these tumors are targets for antiangiogenesis drug treatment. Expression of the *mdr-1* gene is proposed to be an indicator of multidrug resistance (38–40). Poor response to endocrine therapy has been indicated for urokinase-type plasminogen activator/plasminogen activator inhibitor-1, a plasminogen activator inhibitor (21). Also receiving major attention are the familial breast cancer-related genes *BRCA1* and *BRCA2* (40–42). With the availability of tens of thousands of EST sequences, we have, using differential cDNA sequence, identified a new putative breast cancer marker gene, *BCSG1*, and studied its expression in breast cancer.

The differential cDNA sequencing method described here is a direct approach that utilizes an automatic EST analysis on a pair of cDNA libraries. Unlike previously described methods, the differential cDNA sequencing approach allows one to identify differentially expressed genes or patterns of genes directly from a computer database. With the advancement of more efficient and rapid sequencing technology, the direct differential cDNA sequencing approach may offer a powerful method for simultaneous analysis of the expression profile of thousands of genes, as well as for the discovery of novel genes of clinical interest.

Using *in situ* hybridization analysis, we have demonstrated the expression of BCSG1 transcripts in the neoplastic epithelial cells of infiltrating breast carcinoma but not in epithelial cells of normal and benign breast. The overexpression (17 of 20) of BCSG1 in malignant infiltrating breast epithelial cells compared to the partial expression (7 of 15) in *in situ* carcinoma suggests that up-regulation of BCSG1 expression is associated with breast cancer malignant progression and may signal the more advanced invasive/metastatic phenotype of human breast cancer. This implication is supported further by the detection of BCSG1 expression in six of eight aggressive Comedo-type DCISs and in only one of seven non-Comedo type DCISs. It is unlikely that BCSG1 is overexpressed as a secondary effect of cellular proliferation, because no detectable BCSG1 expression is evident in rapidly proliferating nonmalignant breast lesions (Fig. 4*F*).

It will be interesting to investigate whether BCSG1 expression in DCIS may indicate a malignant progression leading to invasion and metastasis. There is cause for concern about the large number of DCIS cases that are being diagnosed as a consequence of screening mammography, most of which are treated by some form of surgery. In addition, the proportion of cases treated by mastectomy may be inappropriately high (30). DCIS by definition has intact basement membrane by light microscopy (43). Defective basement membranes, however, have been found when they are stained with periodic acid-Schiff reagent and when they are examined by electron microscopy (44). In fact, it has been reported that re-evaluation by experienced pathologists showed that 28 and 15% of previously diagnosed DCISs demonstrated invasion (45, 46). If BCSG1 expression can provide some prognostic information on distinguishing the DCIS that is not likely to become invasive from the DCIS that is most likely to become invasive, this will help to direct the treatment strategies and to reduce some inappropriate or unnecessary mastectomies.

It is interesting to note that the predicted amino acid sequence of *BCSG1* gene shares a high sequence homology with the non-Aβ component of the AD amyloid precursor protein (28). A neuropathological hallmark of AD is a widespread amyloid deposition resulting from β-amyloid precursor proteins. β-Amyloid precursor proteins are large, membrane-spanning proteins that either give rise to the β-A4 peptide (Aβ fragment; Ref. 47) or a non-Aβ component of AD amyloid (28) that is either deposited in AD amyloid plaques or yielding soluble forms. Although the insoluble membrane-bound AD amyloid destabilizes calcium homeostasis and thus renders cell vulnerable to excitotoxic conditions of calcium influx resulting from energy deprivation or overexcitation (48), the soluble AD amyloid proteins are neuroprotective against glucose deprivation and glutamate toxicity, perhaps through their ability to lower the intraneuronal calcium concentration (49). We currently do not know whether BCSG1 is an instigator or a by-product during breast cancer progression. With the availability of anti-BCSG1 antibody to localize BCSG1 protein and the recombinant BCSG1 protein, we may start to speculate that BCSG1, like soluble AD amyloid, may be potentially involved in protection from tissue damage resulting from tissue remodeling due to the local cancer invasion. An elucidation of the reasons for BCSG1 overexpression in infiltrating breast cancer cells may shed some light on the pathogenesis of breast cancer progression. Nevertheless, we demonstrated a stage-specific BCSG1 expression and an association of BCSG1 overexpression with clinical aggressiveness of breast cancers. The notion that the BCSG1 overexpression may indicate breast cancer malignant progression from benign breast or *in situ* carcinoma to the highly infiltrating carcinoma warrants further investigation.

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Stimulation of breast cancer invasion and metastasis by synuclein γ (SNCG)

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Running title: SNCG in human breast cancer metastasis

Abbreviations: AD, Alzheimer's disease; APMA, p-aminophenylmercuric acetate; BCSG1, breast cancer specific gene; EST, expressed sequence tag; NAC, non-Aß component of AD; MAP, microtubule-associated protein; MMP, matrix metalloproteinase; PD, Parkinson's disease; SNCA, synuclein α; SNCB, synuclein β; SNCG, synuclein γ; TIMP, tissue inhibitor of metalloproteinase.

Abstract

We recently identified and cloned a novel breast cancer-specific gene BCSG1 by direct differential cDNA sequencing (Cancer Res., 57: 759-764, 1997). BCSG1 has a great sequence homology with Alzheimer disease (AD)-related neurotic proteins synuclein α (SNCA) and synuclein β (SNCB), and thus was also named as synuclein γ (SNCG). To determine if SNCG can modulate the tumorigenic and metastatic phenotypes of human breast cancers, we transfected a full-length SNCG cDNA into MDA-MB-435 human breast cancer cells and studied the orthotopic growth and metastasis in the athymic nude mice. Overexpression of SNCG in breast cancer cells led to a significant increase in motility and invasiveness *in vitro* and a profound augmentation metastasis *in vivo*. Our data suggest that the class of neurotic protein synucleins might have important functions outside the central nervous system and play a role in breast cancer progression.

Introduction

Identification of quantitative changes in gene expression that occur in the malignant mammary gland, if sufficiently characterized, may yield novel molecular markers which may be useful in the understanding of breast cancer development and progression (1). Within this context, we have previously reported the isolation of differentially expressed genes in the cDNA libraries from normal breast and infiltrating breast cancer using the EST-based differential cDNA sequencing approach (2, 3). Of many putative differentially expressed genes (2, 3), a breast cancer specific gene, BCSG1, which was (a) identified as a group of EST specifically expressed in mammary gland relative to other organs and was (b) high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified as a putative breast cancer specific gene (2).

Interestingly, BCSG1 revealed no homology to any other known growth factors or oncogenes; rather, BCSG1 revealed extensive sequence homology to AD-related neurotic proteins synucleins that are mainly expressed in brain and localized to presynaptic terminals (4-7). The pathological hallmark of AD is amyloid deposition in neurotic plaques and blood vessels (8). Two major intrinsic constituents of amyloid are a 39-43 AA peptide named Aß component (8) and the recently identified non-Aß component (NAC) (4). NAC precursor was cloned from a human brain library (4) and named synuclein α (SNCA) because it shares 95% sequence homology with rat synuclein. Recently, the second synuclein named synuclein ß (SNCB) was cloned from human brain and has 61% identical sequence with SNCA (6). The previously identified BCSG1, which is also highly expressed in brain (2), has 54% and 56% sequence identity with SNCA and SNCB, respectively, and has been renamed as synuclein γ (SNCG) (9). Thus, the previously unrecognized homology between these proteins defines a family of human brain synucleins that currently has three members. Although synucleins are abundant proteins expressed in presynaptic terminals and tightly associated with amyloid plaque in AD and Lewy body in Parkison's disease (PD) (10), their functions have not been defined yet. SNCA aggregation may be important in the etiology and pathogenesis in neurodegenerative disorders such as AD and PD (10). Being identified as a breast cancer specific gene, we previously demonstrated a stage-specific SNCG expression as follows: SNCG was

undetectable in normal or benign breast lesions, showed partial expression in ductal carcinoma in situ, but was expressed at an extremely high level in advanced infiltrating breast cancer. In the current studies, the effects of SNCG on breast cancer growth and metastasis were investigated.

Methodology

Transfection. The full-length SNCG cDNA was inserted into a pCI-neo mammalian expression vector and the resulting vector was transfected into MDA-MB-435 cells as we described previously (3, 10).

Preparation of conditioned media (CM). All the clones were maintained in subconfluent monolayers with 10% fetal calf serum. The medium was discarded and the monolayers washed twice with phosphate-buffered saline (PBS). The monolayers were cultured in the absence of serum, in DMEM supplemented with transferrin (1 mg/L), fibronectin (1 mg/L), and trace elements (Biofluides, Rockville, MD). After 24 hours, the serum-free medium was discarded, and the cells were replenished with the fresh serum-free medium. The CMs were collected 30 hours later. Media were then centrifuged at 1,200 x g, and supernatants were saved and concentrated approximately 5-fold using an Amicon hollow fiber concentrator with a 10,000 molecular weight cut off at 4^0C. The protein concentrations of CMs were determined and normalized.

Matrix metalloproteinase (MMP) activity. MMP enzymatic activity of the CMs was assayed using a quenched fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Bachem) as we previously described (28). The CMs were pretreated with APMA for activation (29).

In vitro invasion and motility assay. As we described previously (10), cell invasion and motility were analyzed in the modified Boyden chamber assay with 8-μm polycarbonate membranes coated with 4 mg/ml growth factor-reduced Matrigel.

Tumor growth in athymic nude mice. A nude mouse tumorigenic assay was performed as we previously described (3, 11). Briefly, approximately $0.4x10^6$ cells (0.15 ml) were injected into a 5-6 week old female athymic nude mouse (Frederick Cancer Research and Development Center, Frederick, MD). Each animal received two injections, one on each side, in the mammary fat pads between the first and second nipples. Tumor size was determined at weekly intervals by three-dimensional measurements (mm) using a caliper. Only measurable tumors were used to calculate the mean tumor volume for each tumor

cell clone at each time point. Animals were sacrificed between 32-40 days after injection, when the largest tumors reached about 15 mm in diameter.

Assessment of a regional lymph node and lung metastasis. As we previously descried (11), the axillary lymph nodes and lungs of sacrificed animals were excised, weighted, fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin (H & E) for microscopic examination for morphologic evidence of tumor metastasis. Sections were reviewed and scored by two pathologists.

Antibody Production. The purified synthetic SNCG peptide corresponding to AA 101-117 (2) was conjugated and injected into New Zealand rabbits as we previously reported[28]. The antiserum was purified with a SNCG peptide affinity chromatography.

Results and Discussion.

Transfection of SNCG into MDA-MB-435 human breast cancer cells. In order to determine the effects of SNCG on tumorigenesis and invasion/metastasis, we have selected MDA-MB-435 human breast cancer cells as recipients for SNCG mediated gene transfection because of: 1) their lack of detectable of SNCG transcript (2); and 2) their highly tumorigenic and aggressive phenotype in nude mouse (11). Cells were transfected with a plasmid vector containing a neomycin resistance gene (neo clones), or the same vector containing a full-length of SNCG cDNA (SNCG clones). Clones were initially screened by *in situ* hybridization on slides with a specific SNCG antisense probe (data not shown), and the positive clones were subjected to Northern blot and Western blot analysis. MDA-MB-435 clones expressing SNCG were named as SNCG-435 clones, and the control neo transfected cells were named as neo-435 clones. Fig. 1 shows the Northern blot and Western blot analysis of SNCG expression in selected clones. All selected SNCG-435 clones expressed SNCG mRNA transcripts and proteins. In contrast, none of the neo-435 clones produced any detectable SNCG transcripts and proteins. No changes in morphology were observed in these clones. Based on the level of SNCG expression, we selected SNCG-435-1, SNCG-435-3, neo-435-1, and neo-435-2 clones for the subsequent studies.

In vitro growth of SNCG transfected cells. To determine whether SNCG overexpression affects the growth of MDA-MB-435 cells, cells from exponentially growing cultures of different MDA-MB-435 clones were seeded in triplicate at 3,000 cells per well (24-well plate) in 1 ml DMEM-5% serum. The growth rates of SNCG positive SNCG-435-1 and SNCG-435-3 cells were compared to that of SNCG negative neo-435-1 and neo-435-2 in a monolayer culture. No significant differences in growth rate were observed among SNCG positive and SNCG negative cells (data not shown).

Metastasis in orthotopic nude mice model. Since SNCG was highly expressed in the infiltrating breast cancer cells relative to benign or non-invasive *in situ* carcinomas (2), we are interested in studying whether SNCG is an instigator of metastasis or merely a correlative product during breast cancer progression. The effect of SNCG expression on metastasis was assayed in an *in vivo* orthotopic (mammary fat pad) nude mouse model. Two independent experiments were done to confirm

reproducibility, and the data from these experiments are summarized in Table 1. After a lag phase of 10 days, mice given implants of both SNCG positive and SNCG negative cells developed tumors. There was no difference in tumor incidence between neo-435 and SNCG-435 clones. Starting at about 20 days after inoculation, tumor necrosis was observed in tumors derived from SNCG-435-1 and SNCG-435-3 cells. Neo-435-1 and neo-435-2 also developed some tumor necrosis but with less intensity. Consistent with the *in vitro* similar growth rates, at the end of 40 days after injection, there was no significant difference in primary tumor size between neo-435 and SNCG-435 clones.

To study tumor dissemination, axillary lymph nodes and lungs were first examined physically at autopsy and then subjected to microscopic examination for morphologic evidence of tumor cells by light microscopy on H & E stained paraffin sections. For the axillary lymph node, the average weight was 15 mg for neo-435 mice, and was increased to 44 mg for SNCG-435 mice. The increased lymph node weight reflects the invaded breast tumors. A representative H & E stained sections for neo-435 and SNCG-435 lymph nodes are presented (Fig. 2). Microscopic examination indicated that SNCG-435-1 and SNCG-435-3 mice showed an average significantly higher lymph node positivity of 64% and 77% as compared to the average of 27% from SNCG negative neo-435-1 and neo-435-2 mice (Table 1). For the lung metastasis, the numbers of visible nodules on the surface of the lungs were dramatically increased from an average of 1 for neo-435 mice to the average of 23 for SNCG-435 mice (Table 1). The representative lungs were shown in Fig. 3. When these lungs were examined microscopically, large numbers of micro-metastasis were observed in SNCG-435 mice; the lungs from neo-435 mice had significantly less micro-metastasis (data not shown). A representative H & E stained sections for neo-435 and SNCG-435 lungs are presented (Fig. 4). To our knowledge, human breast cancer cells usually do not form such profound regional and metastatic tumor dissemination (visible lung nodules) in the spontaneous mammary fat pad nude mouse model. This dramatic SNCG-stimulated metastasis suggests a role of SNCG as one of key positive regulator for breast cancer invasion and metastasis. The overexpression of SNCG in malignant infiltrating breast epithelial cells compared to the low level expression in the non-invasive in situ carcinoma (2) suggests that SNCG expression is a meaningful marker for breast cancer malignant

progression and may signal the more advanced invasive/metastatic phenotype of human breast cancer. In this regard, the up-regulation of SNCG expression may facilitate the breast cancer progression leading to metastasis.

Matrix metalloproteinase (MMP) activity. In an effort to investigate the molecular mechanisms underlying SNCG-induced metastasis, we studied several invasion-related factors including MMP and cell motility. The amyloid protein has been recently demonstrated to be a strong stimulator for MMP-2 and MMP-9 expression in astrocytes (12). It is well established that the overproduction and unrestrained activity of MMPs and particular MMP-2 and MMP-9 has been linked to malignant conversion of a variety of different tumor cells (13-20) including mammary tumors (16-20). It is interesting to test whether SNCG, an amyloid-related protein, stimulates MMP-2 and MMP-9 expression in breast cancer cells and leads to the more metastatic phenotype. We investigated if the SNCG overexpression would increase the MMP activity of MDA-MB-435 cells. In this regard, the pooled conditioned media (CM) from two SNCG negative cells and the pooled CM from two SNCG positive cells were concentrated and subjected to MMP enzymatic assay. As shown in Fig. 5, no significant differences of basal levels proteolytic activities were observed between neo-435 and SNCG-435 clones. Mammalian MMPs are usually secreted as latent proenzymes (zymogen) and require activation for their enzymatic activity. Incubation of the CMs with MMP activator organomercurial compounds APMA resulted in approximately 2-fold increase in proteolytic activity for CMs from both neo-435 and SNCG-435 clones. However, no significant difference on APMA-activated MMP activities was observed between neo-435 and SNCG-435 clones. Since the measured enzymatic activity represents the net MMP activity reflecting the balance between activated MMPs and the tissue inhibitors of metalloproteinase (TIMPs), our data suggest that SNCG-induced metastasis may not be mediated by regulation of MMP and TIMP.

Stimulation of invasiveness and motility of MDA-MB-435 cells by SNCG. We used an *in vitro* reconstituted basement membrane (Matrigel) invasion assay to determine the effect of SNCG on cell invasion. All three SNCG negative cells parental MDA-MB-435, neo-435-1, and neo-435-2 cells were moderately invasive. At the end of 48-h incubation, approximately average 250 of SNCG negative cells

had crossed the Matrigel barrier. A significant stimulation in invasiveness was noted in two SNCG positive clones with a 3-fold increase for SNCG-435-1 cells and a 4.3-fold increase for SNCG-435-3 cells (Fig. 6A). We also investigated the effect of SNCG on cell migration (without Matrigel). A similar SNCG-stimulated pattern on migration was observed. At the end of 24-h incubation, SNCG-435-1 cells migrated 4-fold and SNCG-435-3 cells migrated 4.2-fold over that of average SNCG negative cells (Fig. 6B). The similar magnitude of the invasion-stimulating and migration-stimulating activity of SNCG suggests the increased invasion in SNCG clones may be mediated by alternation of cell motility. In order to determine whether the increased cell motility is mediated by chemotaxis due to the different concentrations of serum or chemoattractants in the top and bottom chambers, we picked up SNCG-435-3 and neo-435-1 cells and compared their migration at three different culture conditions: serum free, serum with gradient, and serum without gradient. As shown in Fig. 7, although the migration was relative low in the serum free conditions, there was a 2.8-fold increase in migration in SNCG-435-3 cells compared with that in neo-435-1 cells. When 2% serum was added in the top chamber, the migration of both SNCG positive and negative cells increased significantly. However, the migration of SNCG-435-3 cells was not affected by serum gradient. Approximately 1,600 SNCG-435-3 cells migrated into the bottom chamber contained either same 2% serum or 10% serum. These data suggest that the increased migration in SNCG positive cells is not likely to be mediated by chemotaxis but rather by cellular intrinsic high motile features.

Many breast tumors go through a series of events from the time of initial detection to the formation of the lethal invasive and metastatic stage. The ability of the tumor cells to cross the basement membrane of epithelial barriers it encounters during metastasis is believed to be a dominant factor in its invasive potential. According to the three-step hypothesis of invasion (21), cell adhesion, local proteolysis and subsequent migration or motility are key steps to traversal of basement membrane and connective tissue. In the present report, we provided evidences linking overexpression of a neurotic protein SNCG, a previously identified breast cancer specific gene (2), in human breast cancer cells with increased motility and invasive activity *in vitro* and a profound augmentation of metastasis *in vivo*.

Synuclein proteins share structural resemblance to apolipoproteins, but are abundant in the neuronal cytosol and present in enriched amounts at presynaptic terminals (9). Synucleins have been specifically implicated in two diseases: AD and PD. In AD patients, a peptide derived from SNCA forms an intrinsic component of plaque amyloid (9). In PD patients, an SNCA allele is genetically linked to several independent familial cases, and the protein appears to accumulate in Lewy bodies (9). We currently do not know the significance of the involvement of a neurotic protein SNCG in cancer metastasis in general. Recently, SNCA and SNCB were identified as two abundant proteins through their reactivity on immunoblots with a monoclonal antibody recognizing microtubule-associated protein (MAP) tau (6). In eukaryotic cells, microtubules, actin, and intermediate filaments interact to form the cytoskeletal network involved in determination of cell architecture, mitosis, differentiation and motility (22). Cytoskeletal organization and dynamics depend on protein self-associations and interactions with regulatory elements such as MAPs. There is increasing evidence that microtubule-associated proteins including tau play a critical role in inducing microtubule assembly and in controlling the dynamic instability of microtubules, thus controlling the state of their assembly and organization in cells (reviewed in ref. 22). SNCG may interact with MAPs, regulate the cytoskeletal organization and dynamics, and thus lead to increased motility. Nevertheless, our data indicate that the increased expression of SNCG correlates with breast progression (2) and leads to more malignant metastatic phenotype. We also demonstrated that SNCG expression in breast cancer cells was subjected to cytokine regulation and was dramatically suppressed by tumor growth inhibitor oncostatin M (OM) and this OM-induced transcriptional suppression of SNCG gene was associated with OM-mediated growth inhibition (23). OM is an anti-tumor cytokine mainly produced by activated T cells and macrophages (24) and its growth suppressing activity was well studied in breast cancer cells (25-27). One characteristic of the host response to tumor progression is the infiltration of tumors by macrophages and T lymphocytes. Production of tumor suppressing cytokines in a timely and locally (in situ) released fashion may represent an important function of the host defense system to suppress tumor progression. From this prospective view, the dramatic suppression of SNCG

expression in malignant breast cells by OM may represent the host-mediated tumor suppression leading to the inhibition of breast cancer progression.

This is the first report indicating the potential involvement of synuclein in the non-neurotic disease. An elucidation of the reasons for SNCG overexpression in infiltrating breast cancer and SNCG-induced metastasis may shed some light on the pathogenesis of not only breast cancer progression but also neurodegenerative disorders.

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Figure Legends

Fig.1. Transfection of SNCG to MDA-MB-435 cells. A. Northern blot. Each lane contained 30 μg total RNA. B. Western blot with an affinity purified specific SNCG peptide polyclonal antibody. Each lane contained 20 μg protein. 1, neo-435-1. 2, SNCG-435-2. 3, SNCG-435-1. 4, SNCG-435-3. 5, neo-435-2.

Fig. 2. Axillary lymph nodes from neo-435 mice and SNCG-435 mice. The mice were sacrificed at day 40 after cell injection. Lymph nodes were isolated and subjected to H & E staining. Representatives of axillary lymph nodes from neo-435-1 mouse (A) and SNCG-435-3 mouse (B) are presented. An arrow indicates invaded breast tumor that mainly occupied the lymph node from SNCG-435-3 mouse.

Fig. 3. Representative lung metastases from mice injected with SNCG positive and SNCG negative cells. Cell injection and tumor formation were described in Methodology. Left lung was from neo-435-1 mouse and right lung was from SNCG-435-3 mouse. The metastatic tumors only reflect the nodules on the surface of the lungs.

Fig. 4. Microscopic examination of representative lung metastasis in H & E stained sections. A, a lung without metastasis from neo-435-1 mouse. B, a lung with micro-metastasis from neo-435-2 mouse. C, a lung with a small breast tumor nodule from SNCG-435-1 mouse. D, a lung with a big breast tumor nodule from SNCG-435-3 mouse. Arrows indicate the breast tumors or cancer cells.

Fig. 5. Analysis of MMP activities of SNCG positive and SNCG negative cells. The pooled CMs from SNCG negative neo-435-1 and neo-435-2 cells and SNCG positive SNCG-435-1 and SNCG-435-3 cells were collected, concentrated 5-fold, normalized for protein concentrations, and subjected to MMP activity analysis. Recombinant AMPA-activated MMP2 (80 ng) was used as a positive control. All values were normalized to the enzymatic activity of the recombinant MMP2 which was taken as 100%. The numbers

represent the means ± SD of three tests.

Fig. 6. Stimulation of invasiveness and migration of MDA-MB-435 cells by SNCG. Cells were seeded at a density of 30,000 cells/ml/well on the 8-μm polycarbonate membranes coated with (A) or without (B) 4 mg/ml growth factor reduced Matrigel. The top chamber contained 5% FCS and the bottom chamber contained 10% FCS. (A) Following an incubation in a humidified incubator with 5% CO_2 at 37^0C for 48 hrs, the medium as well as the cells were removed from the bottom chambers and counted using a microscope. (B) Cells were cultured in the same conditions as in A. The numbers of migrated cells were counted after 24 hrs incubation. All values were expressed as the number of invaded cells. The numbers represent the means ± SD of three cultures.

Fig. 7. Comparison of cell migration of SNCG-435-3 and neo-435-1 cells at different conditions. Cells were cultured on the non-coated membrane at the density of 30,000 cells/ml/well. The migrated cells were harvested at 32 hr after incubation. A, 0% serum in both top and bottom chambers. B, 2% serum in both top and bottom chambers. C, 2% serum in top chamber and 10% serum in bottom chamber. All values were expressed as the number of invaded cells. The numbers represent the means ± SD of triplet wells.

Acknowledgments

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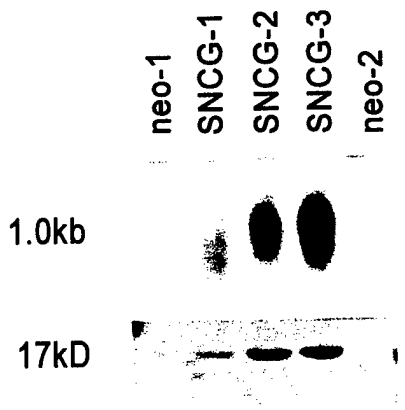


Figure 1

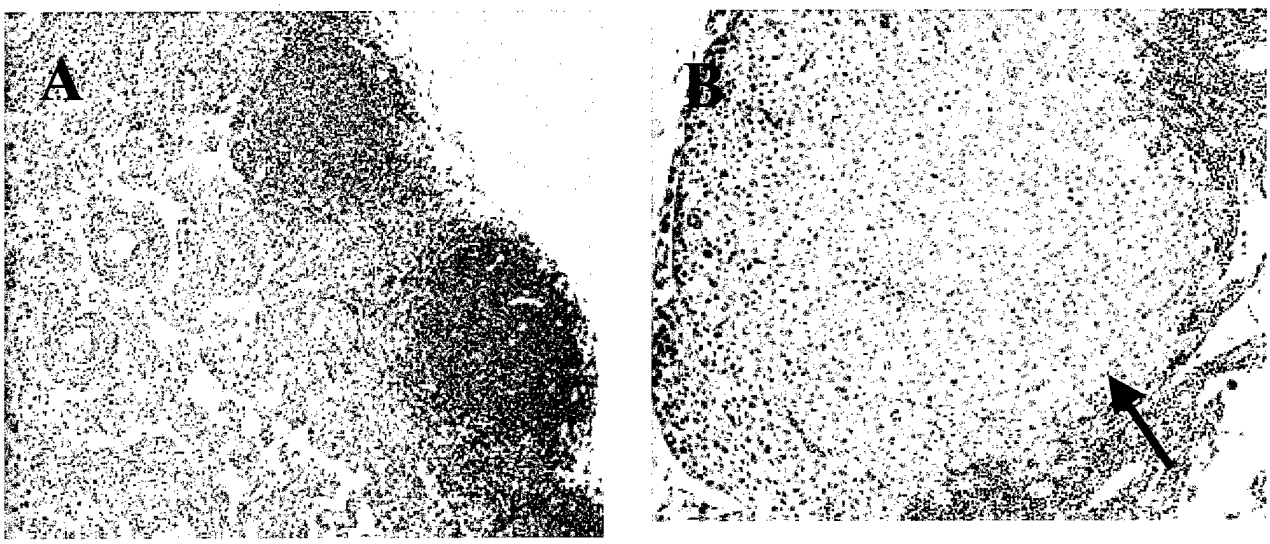


Figure 2

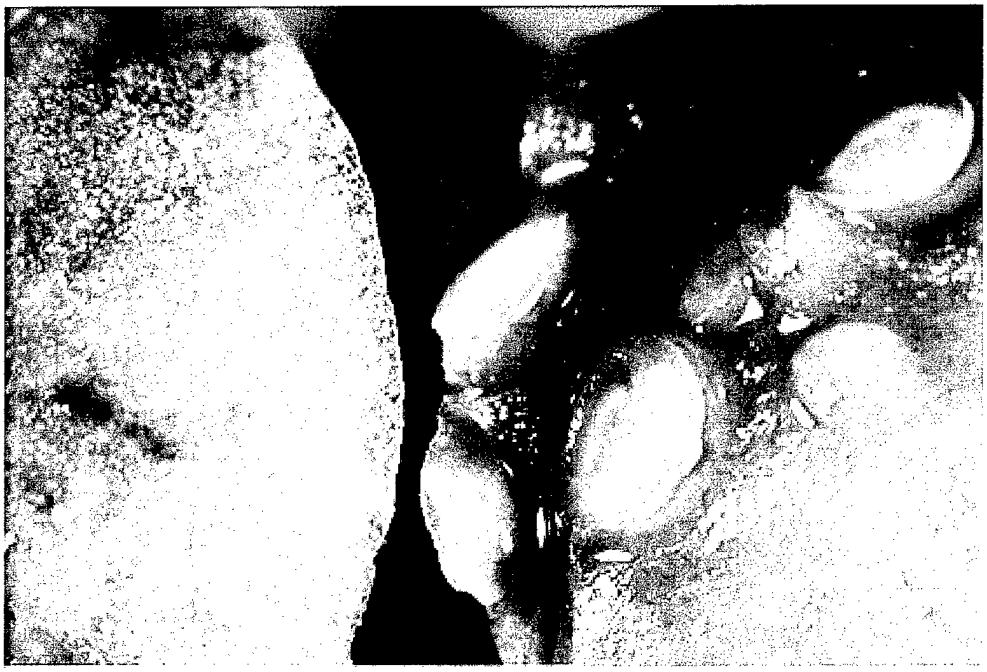


Figure 3

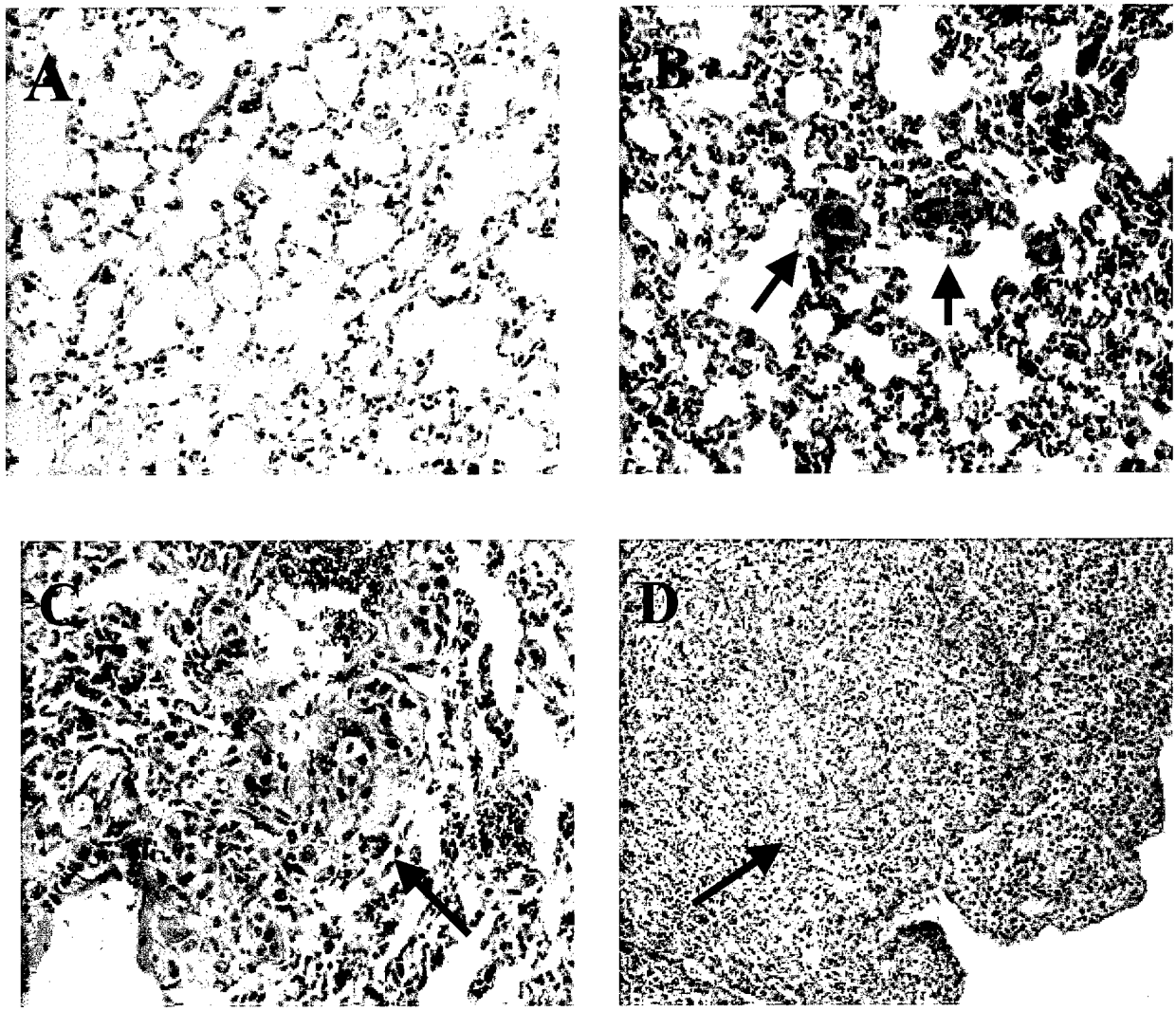


Figure 4

Fig. 5

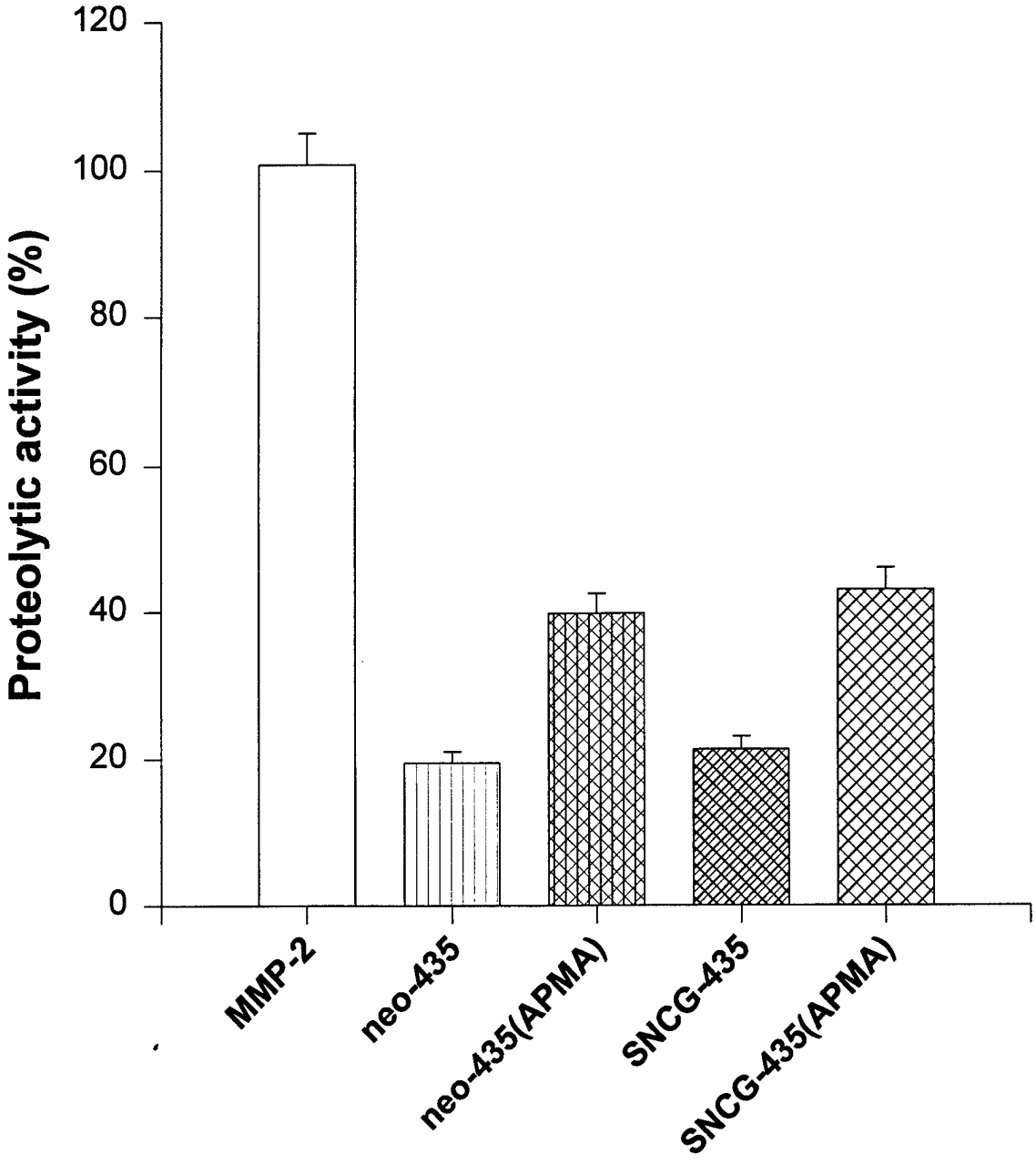
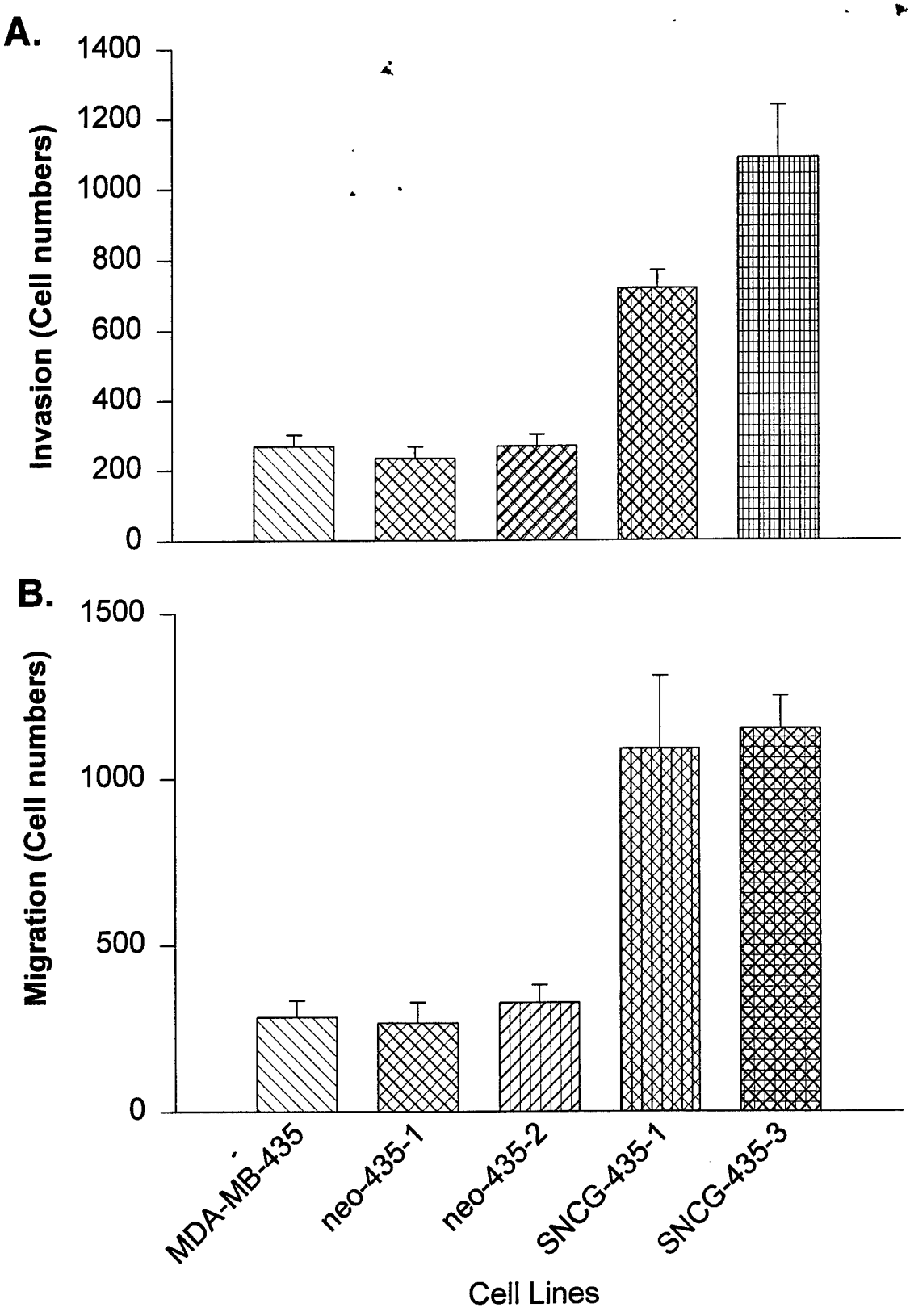

Proteolytic activity (%)
120
100
80
60
40
20
0
MMP-2
neo-435
neo-435(APMA)
SNCG-435
SNCG-435(APMA)

Fig. 6


A.
Invasion (Cell numbers)
1400
1200
1000
800
600
400
200
0
B.
Migration (Cell numbers)
1500
1000
500
0
MDA-MB-435
neo-435-1
neo-435-2
SNCG-435-1
SNCG-435-3
Cell Lines

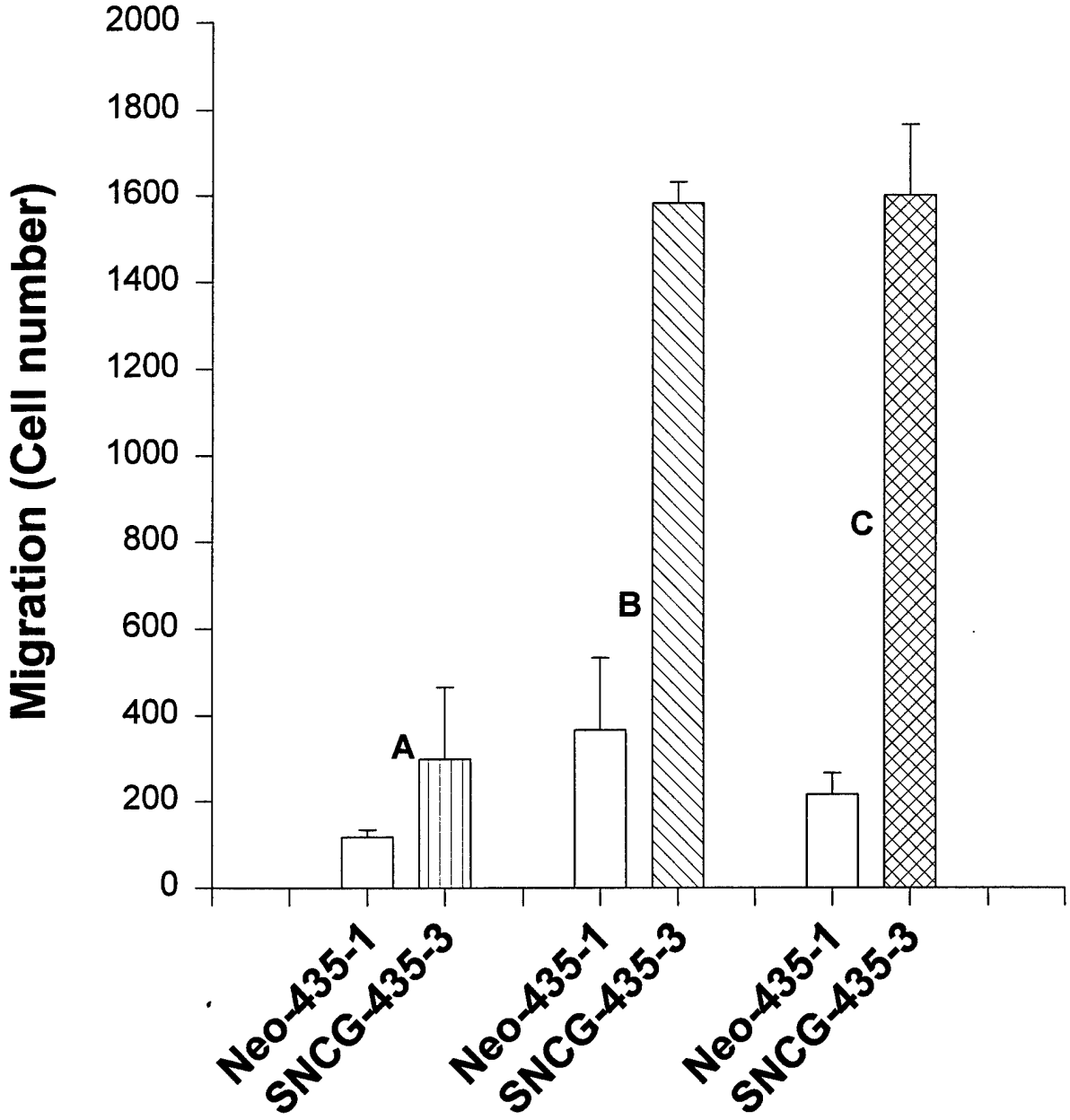

Migration (Cell number)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
A
B
C
Neo-435-1
SNCG-435-3
Neo-435-1
SNCG-435-3
Neo-435-1
SNCG-435-3

Figure 7

Table 1. Effects of SNCG on tumor incidence, tumor size, axillary lymph node, and lung metastasis

Experiment	Clones	Tumor Vol(cm^3) of Primary Size	Tumor Incidence Tumor/Total (%)	Lymph node Average weight	Lymph node Positive/total (%)	Lung metastasis Number of nodule
1	neo-435-1	1.74 ± 0.52	16/16 (100)	14 mg	3/16 (19)	0
	neo-435-2	1.9 ± 0.31	14/16 (88)	18 mg	4/15 (27)	2
	SNCG-435-1	1.45 ± 0.37	15/16 (94)	43 mg	10/15 (67)	19
	SNCG-435-3	1.78 ± 0.31	16/16 (100)	50 mg	12/16 (75)	31
2	neo-435-1	1.35 ± 0.39	9/10 (90)	12 mg	3/10 (30)	1
	neo-435-2	1.69 ± 0.44	10/10 (100)	15 mg	3/9 (33)	1
	SNCG-435-1	1.73 ± 0.45	10/10 (100)	45 mg	6/10 (60)	24
	SNCG-435-3	1.49 ± 0.34	10/10 (100)	39 mg	7/9 (78)	17

Four hundred thousand of the cells were injected at day one into the mammary fat pads, and tumor volumes and lymph node and lung micro-metastasis were determined. Lymph node metastases were measured by microscopic examination for morphologic evidence of tumor cells on fixed axillary lymph nodes. Lung metastases were measured by the presence of number of visible tumor nodules on the surface of the lung examined. Volumes are expressed as means ± SEs (number of tumors assayed). Experiment 1, total 16 injections for 8 mice in each group, and the mice were sacrificed 42 days after injection. For experiments 2, total 10 injections for 5 mice for each group, and the mice were sacrificed 38 days after injection. Statistical comparisons for SNCG positive clones relative to SNCG negative clones: there was no significant difference for the mean tumor sizes between pooled SNCG positive and pooled SNCG negative tumors; lymph node positivity of pooled SNCG-435-1 tumors vs. combined pooled SNCG negative neo-435-1 and neo-435-2 tumors gave $P < 0.039$; $P < 0.029$ for pooled SNCG-435-3 tumors vs. SNCG negative tumors. Statistical comparison for primary tumors was analyzed by Student's *t* test. A chi-squared test was used for statistical analysis of lymph node metastasis.

Title

Transcriptional suppression of the breast cancer-specific synuclein γ (*SNCG*) expression by the growth inhibitory cytokine oncostatin M

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Running Title
Transcriptional suppression of *synuclein γ* by oncostatin M

Key Words
Synuclein proteins, Breast cancer progression, Transcriptional regulation, Cell growth, Oncostatin M

FOOTNOTES:
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3. The abbreviations used are:

AD	=	Alzheimer's disease
BCSG1		breast cancer specific gene 1
CNTF	=	ciliary neurotrophic factor
CT-1	=	cardiotrophin-1
FBS	=	fetal bovine serum
GAPDH	=	glyceraldehyde-3-phosphate dehydrogenase
IL-6	=	interleukin 6
IL-11	=	interleukin 11
IMDM	=	Iscoves modified Dulbecco's medium
LIF	=	leukemia inhibitory factor
OM	=	oncostatin M
SNCA	=	synuclein α
SNCB	=	synuclein β
SNCG	=	synuclein γ

ABSTRACT

Recently, a novel breast cancer specific gene designated *BCSG1*, was isolated from a human breast cancer cDNA library by the differential cDNA sequencing approach. *BCSG1* has a great sequence homology with Alzheimer disease (AD)-related neurotic protein synuclein α (SNCA) and synuclein β (SNCB), and thus was also named as synuclein γ (*SNCG*). The mRNA of *SNCG* is highly expressed in the infiltrating carcinomas but not expressed in normal or benign breast tissues. We have detected the mRNA of *SNCG* in several breast cancer cell lines with the highest expression found in H3922, a cell line derived from an infiltrating ductal carcinoma. To investigate whether *SNCG* gene expression is related to cell growth, we examined the effect of oncostatin M (OM) on *SNCG* gene expression, as OM strongly inhibits the growth of these cells. We found that *SNCG* mRNA level was marked decreased in OM-treated cells. A kinetic study showed that treatment of H3922 cells with OM initiated an immediate decrease of *SNCG* mRNA as early as 30 min. By 4 h, the level of *SNCG* mRNA was decreased to 70% of control, and by 24 h, the mRNA was completely undetectable. Results from nuclear run-on analysis and mRNA stability studies indicated that the observed OM-induced down-regulation of *SNCG* mRNA occurs mainly at the transcriptional level. To further investigate the role of *SNCG* gene in the proliferation of breast cancer cells, *SNCG* cDNA was stably transfected into MCF-7 cells that do not express endogenous *SNCG* gene. Cell proliferation assay show that over expression of *SNCG* gene stimulated the growth of MCF-7 cells up to 3.2 fold over muck-transfected cells. Our study suggests that the neurotic protein *SNCG* may have important functions outside the central nervous system and may play a role in the growth of breast cancer cell.

INTRODUCTION

Breast cancer development and progression is accompanied by multiple genetic changes that lead to qualitative and quantitative alterations in individual gene expression. Consequently, the altered levels of these gene products and their cellular functions will disturb the normal physiological homeostasis of the cells and result in cancer formation. Identification genes that are over expressed or under expressed in tumors and subsequent evaluation of their biological functions will help to understand the process of malignant transformation. By utilizing a high-throughout direct-differential cDNA sequencing approach, a novel breast cancer specific gene designated *BCSG1,* was recently isolated from a breast tumor cDNA library (1). The *BCSG1* gene is transcripted into a 1kb mRNA, and the open reading frame of the full length gene is predicted to encode a 127-amino acid polypeptide.

Comparison of the predicted amino acid sequence with genetic database reveals that *BCSG1* is highly homologous to Alzheimer's disease-related neurotic protein synucleins that are mainly expressed in brain and localized to presynaptic terminals (2-5). Previously, there are two synuclein proteins have been described, synuclein α (SNCA) and synuclein β (SNCB). SNCA is the precursor of the non-Aß fragment of human AD amyloid protein (2). Since *BCSG1* has 54% and 56% sequence identity with SNCA and SNCB, it is also highly expressed in brain (1), *BCSG1* now is renamed as synuclein γ (*SNCG*) (6). Thus, presently, the human synuclein family has three members, however, only the *SNCG* was found to express in breast cancer cells (take off).

Being identified as a breast cancer specific gene, *SNCG* mRNA was detected exclusively in neoplastic epithelial cells. *In situ* hybridization analysis has demonstrated a stage-specific expression pattern of *SNCG* mRNA varying from virtually no detectable expression in normal or benign breast tissue to low level and partial expression in low grade *in situ* breast carcinoma to high expression in advanced infiltrating carcinomas. This implies that *SNCG* may play a role in breast cancer malignant progression. This implication is supported by analysis of *SNCG* mRNA

in several breast cancer cell lines. Northern blot analysis detected a 1 kb transcript corresponding to *SNCG* in 2/4 human breast cancer cell lines derived from pleural effusions and 4/4 breast cancer cell lines derived from ductal infiltrating carcinomas (1).

The high level expression of *SNCG* in the malignant breast epithelial cells suggest that the expression of *SNCG* may be up-regulated in the mammary gland during the onset and progression of breast cancer. We initiated a study to investigate whether the gene expression of *SNCG* can be regulated by factors that affect the growth and differentiation of breast cancer cells. Previously we demonstrated that H3922 cells, a breast cancer cell line derived from a ductal infiltrating carcinoma, express high level of *SNCG* mRNA, and that the cellular proliferation of H3922 cells was inhibited by cytokine oncostatin M (OM) (7,8).

OM is a 28kDa glycoprotein produced by activated T lymphocytes and monocytes (9-11). OM is a member of the interleukin-6 (IL-6) family of cytokines, which includes IL-6, interleukin-11 (IL-11), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and cardiotrophin-1 (CT-1) (12-14). As a pleiotropic cytokine, OM elicits a number of different biological functions in different cell types. Notable among those is its ability to regulate cell growth and differentiation. OM stimulates the growth of normal fibroblasts (15), normal rabbit vascular smooth muscle cells (16), human myeloma cells (14), and AIDS-related Kapossi sarcoma cells (17,18). OM also has been shown to inhibit the proliferation of a number of cell lines derived from human tumors including melanoma, breast carcinoma, and lung carcinoma (7,8, 15,19).In this study, we have examined the effect of OM on *SNCG* expression and the relationship between *SNCG* expression and cell proliferation.

MATERIALS AND METHODS

Cells and Reagents. The human breast cancer cell line H3922 was developed from a ductal infiltrating breast carcinoma at the Bristol-Myers Squibb Pharmaceutical Research Institute-Seattle. Cells were cultured in Iscoves Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Human recombinant OM was expressed by Chinese hamster ovary cells and purified as previously described (20). The other growth factors and cytokines were obtained from R&D Systems, Minneapolis, MN. The plasmid containing the cDNA probe for *c-Myc* was obtained from American Type Culture Collection (Bethesda, MD).

Northern blot Analysis. Total cellular RNA was isolated by the method of Peppel and Baglioni (21). Approximately 20 μg of each total RNA sample was separated on a 1% formaldehyde agarose gel. RNA was capillary transferred to a Hybond N membrane before crosslinking to the membrane. Prehybridization and hybridization steps were performed under the conditions previously described (7,8). The blot was hybridized at 60° C to a 0.55Kb ^{32}P-labeled human *SNCG* cDNA probe. The probe was labeled using 50 μCi [α-^{32}P] dCTP with random primed DNA labeling kit (Boehringer Mannheim Corp, Indianapolis, IN). The membrane was then washed 3 times at ambient temperature with 2X SSC, 0.1% SDS and twice at 37° C with 0.1X SSC, 0.1% SDS. The membrane was then dried and exposed to X-OMAT scientific imaging film (Kodak, Rochester, NY) for 1-3 days at -80° C. The *c-Myc* and *GAPDH* probes were prepared by random-primer labeling as described for the *SNCG* probe. All other steps in analysis of the membrane were also followed as described for the *SNCG* probe. The autoradiographs were scanned by a laser densitometer (Personal Densitometer^{TM} SI, Molecular Dynamics, sunnyvale, CA) and the integrated intensity of each band was analyzed with the program ImageQuaNT^{TM}, version 4.1. Densitometric analysis of autoradiographs in these studies as well as those discussed below included various exposure times to ensure linearity of signals.

Nuclear Run-on Analysis. These analyses were conducted using a procedure adapted from one that had already been described (8). Briefly, 1.8 x 10^7 adherent H3922 cells were harvested with cell scrapers into a minimal volume of cold phosphate buffered saline (PBS). The cells were pelleted by low-speed centrifugation and lysed with lysis buffer (10 mM Tris-HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40). The nuclei were pelleted by centrifugation and the lysis procedure was repeated once. The nuclei were recovered by centrifugation a second time and resuspended at 10^8 nuclei/ml in glycerol storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA). The samples were immediately frozen under liquid nitrogen and stored at -80°C.

The frozen nuclei were subsequently thawed and 100 µl of each sample received 100 µl 2X reaction buffer (70% glycerol, 0.02 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.16 M KCl, 2 mM DTT, 0.2 mM EDTA, 2 mM rATP, 2 mM rCTP, 2 mM rGTP, 2.6 µCi/µl [^{32}P] rUTP). The reactions were incubated with shaking at 30°C for 30 minutes. Labeled nuclei were pelleted and resuspended with 100 µl DNase buffer (50% glycerol, 20 mM Tris-HCl, pH 7.9, 1 mM $MgCl_2$, 10 mg/ml RNase-free DNase I). The reactions were incubated with shaking at 30°C for 15 minutes. Samples were brought up to 125 µl with 7.5 µl 13.6 mg/ml proteinase K, 5 µl 10 mg/ml yeast tRNA, and 12.5 µl 10X SET buffer (5% SDS, 0.05 M EDTA, 0.01 M Tris-HCl, pH 7.4) and incubated at 42°C for 30 minutes.

Labeled RNA transcripts were extracted by adding the following: 275 µl GCSM solution [4 M guanidinium isothiocyanate, 0.025 M sodium citrate, pH 7.0, 0.5% Sarkosyl, 0.1 M b-mercaptoethanol], 45 µl 2.0 M sodium acetate, 450 µl water-saturated phenol, and 90 µl chloroform:isoamyl alcohol (49:1). The samples were vortexed and incubated on ice for 15 minutes. Nuclear run-on transcripts were precipitated with isopropanol and pelleted by high speed centrifugation. Extractions and isopropanol precipitations were repeated and the samples

were dissolved with 102 µl TES buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% SDS). Assays for radioactivity were conducted by liquid scintillation. Approximately 2.0 x 10^6 cpm of each nuclear run-on reaction was used as a probe to hybridize a Hybond N membrane (Amersham Life Sciences, Arlington Heights, IL) slot blot. Each blot received the following three plasmids: 5 µg plasmid with the human *GAPDH* cDNA insert, 3 µg of the 0.3 kb fragment of the *SNCG* cDNA which is the 3' end of the cDNA. This fragment was generated by cutting the *SNCG* cDNA with the restriction endonuclease BstX1. Probing the *GAPDH* plasmid allowed normalization of the *SNCG* signals measured by densitometry.

Actinomycin D/mRNA stability Analysis. H3922 cells in 100mm tissue culture plates were incubated with or without OM for 6 h. Actinomycin D (5 µg/ml) was added to cells for different lengths of time. At the end of each time point, total RNA was harvested as described above under "Northern Blot Analysis". Electrophoresis of total RNA samples, blotting, and hybridization to radiolabeled probes was also carried out as described above.

Transfection. The full-length *SNCG* cDNA was inserted into a pCI-neo mammalian expression vector. The *SNCG* expression vector (pCI-*SNCG*) and the vector alone (pCI-neo) were transfected separately into MCF-7 cells as we described previously (22). Subsequent to transfection, G418 selection, and cloning by limiting dilution, several subclones of MCF-7 cells were obtained. These G418-resistant clones were expanded into individual cell lines. The *SNCG* mRNA expressions in pCI-*SNCG* transfected cells, but not in muck (pCI-neo) transfected cells were confirmed by northern blot analysis.

Cell Proliferation Assay. Exponentially growing cultures of different MCF-7 clones were detached with trypsin, and the trypsin was neutralized with DMEM with 10% serum. Cells were counted, diluted, and seeded in triplicate at 3,000 cells per well (24-well plate) in 1 ml DMEM with 5% serum. Cell growth was measured using a cell proliferation Assay Kit (Promega, Madison, WI).

RESULTS

Time- and concentration-dependence of OM-mediated suppression of *SNCG* mRNA expression in breast cancer cells.

Breast cancer cell lines MCF-7, ZR-75-1, and H3922 were shown growth-inhibited by OM (7,8,15,19). Because *SNCG* mRNA is not expressed in MCF-7 cells, and is expressed at very low level in ZR-75-1 cells, but expressed at a relatively high level in H3922 cells (1), we examined the effect of OM on *SNCG* mRNA expression in H3922 cells. The results in Figure 1 demonstrated a marked time-dependent suppression of *SNCG* mRNA level by OM. Treatment of H3922 cells with OM initiated an immediate decrease of *SNCG* mRNA as early as 30 minutes. By 4h treatment, the level of *SNCG* mRNA was decreased to 70% of that in control cells and by 24 h the mRNA was completely undetectable. The suppressive effect of OM is persistent, as *SNCG* mRNA was not yet detected in H3922 cells after culture the cells for two days in OM-free medium, *SNCG* mRNA was partially expressed after three days of withdrawing OM from the medium (data not shown).

OM-suppressed *SNCG* transcription was also concentration dependent. After 6h treatment, OM at concentrations as low as 0.2ng/ml caused significant decrease of *SNCG* expression (58% of control), whereas maximal suppressions were observed at concentrations of 1-5ng/ml (Figure 2). This concentration dependence is comparable to those for inhibition of cell growth (7,8,19). These data suggest that inhibition of cell growth and suppression of *SNCG* transcription is correlated.

Transcriptional regulation of *SNCG* expression by oncostatin M.

To determine whether the down regulation of *SNCG* expression by OM occurs at the transcriptional or post-transcriptional level, we conducted nuclear run-on assays to measure the relative transcription rate of *SNCG* in control cells and in the cells treated with OM. As shown in Figure 3, Treatment of H3922 cells with OM for 16 h, decreased the level of actively

transcribed *SNCG* mRNA to 28.5% of that in untreated cells. Data were normalized by the signals observed in the *GAPDH* slots. The level of reduction of *SNCG* transcripts is consistent with the results obtained from northern blot analysis, suggesting a transcriptional regulation mechanism. To confirm this observation, *SNCG* mRNA stability was examined. Control cells and the cells treated with OM for 6 h were exposed to actinomycin D. Total RNAs isolated from the cells at various actinomycin D exposure time points were subjected to northern blot analyses of *SNCG*. (Figure 4). Although OM treatment reduced the level of *SNCG* mRNA to approximately 50% of that in control cells, the *SNCG* mRNA levels in both the control cells and the OM-treated cells were not decreased by actinomycin D. In contrast, the *c-Myc* mRNA levels were rapidly reduced by treatment with actinomycin D. These data suggest that the *SNCG* mRNA stability was not altered by OM, and that the *SNCG* mRNA is relatively stable. An attempt to treat cells with actinomycin D for a longer period of time was not successful due to the actinomycin D-mediated toxicity in H3922 cells. These results together with the data generated from the nuclear run-on assay suggest that *SNCG* gene expression was suppressed by OM mainly at the transcriptional level.

Over expression of *SNCG* gene in breast cancer cells stimulates cell growth

To further investigate the correlation between *SNCG* expression and proliferation of breast cancer cells, we selected MCF-7 cells as a recipient cell line for *SNCG* mediated gene transfection due to its lack of endogenous *SNCG* mRNA expression (1). MCF-7 cells were transfected with a plasmid vector containing a neomycin resistance gene (neo-MCF clones), or the same vector containing a full-length *SNCG* cDNA (*SNCG*-MCF clones). Individual clones were initially screened by *in situ* hybridization on slides with a specific *SNCG* antisense probe, and the positive clones were subjected to northern blot analysis. Fig. 5 shows the northern blot analysis of *SNCG* expression in selected clones. All selected *SNCG*-MCF clones expressed *SNCG* mRNA transcripts. In contrast, none of the neo-MCF clones produced any detectable *SNCG* mRNA. No changes in morphology were observed in these clones.

To determine whether *SNCG* over expression affects the growth of the transfected cells, the growth rates of *SNCG* positive MCF-7 clones (*SNCG*-MCF-2 and *SNCG*-MCF-6) were compared to that of *SNCG* negative MCF-7 cells (neo-MCF-1 and neo-MCF-2) in a monolayer culture. As shown in Figure 6, the cell growth was significantly stimulated in *SNCG* transfected cells compared to muck-transfected cells to approximately 3.2-fold ($p < 0.001$ by Student's *t-test*).

OM-specific receptor type II mediates down regulation of *SNCG* gene expression

The biological activities of OM can be mediated through two types of receptor complexes, the LIF/OM shared receptor (type I) and OSM-specific receptor (OSMR, type II) (23-25). Previous studies conducted in our laboratory showed that the growth-inhibitory activity of OM in the H3922 breast cancer cells is mediated through OM-specific receptor type II (7,26). Although the type I OM receptor that mediates the actions of LIF and OM is also expressed in these cells, LIF did not inhibit the growth of these cells, instead slightly stimulated their growth (7). In addition, previous study showed that IL-6 and IL-11 do not affect the growth of these cells either (7). To determine whether the effect of OM on *SNCG* gene expression is mediated through the type II OM-specific receptor, the effect of LIF, along with IL-6 and IL-11 on *SNCG* expression was compared with OM. H3922 cells were treated with individual cytokines for 24h at a concentration of 100ng/ml. The results of northern blot analysis show that *SNCG* mRNA expression was suppressed by OM, but not suppressed by LIF, or IL-6 and IL-11 (Figure 7). These data suggest that the type II OM specific-receptor transmit OM elicited signals that lead to the repression of *SNCG* transcription.

DISCUSSION

As other synuclein proteins, the normal expression of *SNCG* is restricted to brain tissue (6). Although synucleins are abundant proteins expressed in presynaptic terminals, and SNCA has been found to be a major protein component of the amyloid plaque in Alzheimer's disease and Lewy body in Parkison's disease (5), their functions have not been defined yet. The finding that *SNCG* is highly expressed in advanced infiltrating breast carcinomas, but not expressed in normal breast tissue, suggest that these neurotic proteins might have important functions outside the central nervous system. Furthermore, the over expression of *SNCG* in advanced breast carcinomas suggest that *SNCG* expression might be regulated by factors which play roles in the complex regulation of the growth and progression of breast carcinoma. Indeed, in this report, we have demonstrated that *SNCG* is transcriptionally modulated by the growth-inhibitory cytokine oncostatin M.

We show that *SNCG* mRNA expression is rapidly down regulated by OM. After 24h treatment with OM, *SNCG* mRNA was below the detectable level in H3922 breast cancer cells. Interestingly, the kinetics of OM induced down regulation of *SNCG* is different than the kinetics of OM-induced down regulation of the *c-Myc* gene in these cells. *C-Myc* mRNA was transiently induced by OM within 1 to 4h and subsequently suppressed at later times. The maximal suppression (20-30% of control) occurred after 2 to 3 days of OM treatment at which time the growth was marked inhibited by OM (8). Therefore, the suppression of *SNCG* gene proceeds the inhibition of *c-Myc* gene and cell proliferation. It is an early step in OM-induced cellular events that ultimately lead to cell growth arrest.

The correlative inhibitory effects of OM on cell growth and *SNCG* expression suggest that *SNCG* may be involved in the abnormal growth of breast tumor cells. However, it is important to note that cell growth in general is regulated by a number of different genes, and the gene products exert their effects on cell cycles at different points. It is likely that down regulation of *SNCG* gene expression by OM is part of the process leading to the growth inhibition.

The correlation of the inhibited cell growth and the decreased expression of *SNCG* suggest that *SNCG* might play a role directly or indirectly in the abnormal growth of the breast cancer cells. This hypothesis is supported by the fact that transfection of *SNCG* gene into MCF-7 cells increased the growth rate of the cells 3-fold. Recently, we also show that expression of *SNCG* in breast cancer cell line MDA-MB 435 increased the metastasis of these cells in a nude mice model (27). These data suggest that *SNCG* may be involved in both the growth and the metastasis of the breast cancer cells. Since the normal functions of these synuclein proteins in brain are not yet defined, elucidating the biological activities of *SNCG* and the mechanism for its abnormal expression in breast cancer will provide important clues to understand the pathogenesis of not only breast cancer progression but also neurodegenerative diseases.

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FIGURE LEGENDS

Figure 1. Time-dependent suppression of *SNCG* mRNA expression by OM. Total RNA (20 μg/lane) was isolated from H3922 cells that were cultured in 2% FBS IMEM and treated with OM at a dose of 20 ng/ml for the indicated lengths of time. RNA samples were blotted onto a nylon membrane and hybridized to a ^{32}P-labeled 0.55 Kb *SNCG* cDNA probe as described in "Materials and Methods". The blot was rehybridized under the same conditions with a ^{32}P-labeled human *GAPDH* probe. Radioactive signals were detected by autoradiography and quantified by densitometry.

Figure 2. OM concentration-dependent effects on *SNCG* mRNA expression.
A. Total RNA (20 μg/lane) was isolated from H3922 cells that were untreated (lane 1), 0.2 ng/ml OM-treated (lane 2), 1ng/ml OM-treated (lane 3), 5ng/ml OM-treated (lane 4), 25ng/ml OM-treated (lane 5), or 125ng/ml OM-treated (lane 6). All OM treatments were for 6 h. RNA samples were analyzed as described in Figure 1. **B**. The relative levels of *SNCG* mRNA normalized to *GADPH* mRNA on the autoradiographam shown in panel A were quantified by scanning densitometry. The results shown are representative of two separate experiments

Figure 3. Nuclear runon analysis of *SNCG* transcription. Two slots were blotted onto each of two nylon membrane strips. One slot received 3 μg of the 0.3 kb fragment of the *SNCG* cDNA, which is the 3' end of the cDNA. This fragment was generated by cutting the *SNCG* cDNA with the restriction endonuclease BstX1. The second slot was loaded with 5 μg of the *GAPDH* plasmid. One nylon strip was hybridized to a ^{32}P-radiolabeled nuclear runon reaction prepared from 16-h OM-treated H3922 cells. The second was hybridized to a labeled nuclear runon reaction prepared from control cells. Equal amounts of radioactivity were used in each hybridization. Radioactive signals were detected by autoradiography and quantified by densitometric analysis.

Figure 4. Study of *SNCG* mRNA stability. Cells were either treated with OM at the concentration of 50 ng/ml for 6 h (lane 7-12), or treated with OM dilution buffer, 1 mg/ml BSA in PBS, for the same length of time (lane 1-6), then actinomycin D at a concentration of 5 μg/ml was added to cells for different lengths of time. At the end of each time point, total RNA was harvested and analyzed for *SNCG* mRNA and *c-Myc* mRNA expressions as described in Figure 1. The length of actinomycin D treatment: lanes 1 and 7, 0 h; lanes 2 and 8, 0.5 h; lanes 3 and 9, 1 h; lanes 4 and 10, 2 h; lanes 5 and 11, 3 h; lanes 6 and 12, 4 h.

Figure 5. Northern blot analysis of *SNCG* mRNA expression in transfected MCF-7 cells.
Total RNA was isolated from each clones and 30 μg of total RNA was analyzed. Lane 1, neo-MCF-1; lane 2, SNCG-MCF-2; lane 3, neo-MCF-2; lane 4, SNCG-MCF-6; and lane 5, SNCG-MCF-4.

Figure 6. Cell growth assay of *SNCG* transfected MCF-7 cells. Cells were cultured in 5% FBS DMEM medium with a seeding density of 3000 cells per well in 24 well culture plates. Cell number was determined after culturing cells at the indicated time. The data shown are representative of 5 experiments in which triplicate wells were assayed for each data point.

Figure 7. Comparison of effects of OM related cytokines on *SNCG* gene transcription.
H3922 cells were treated for 24 h with each factors at 100ng/ml concentration respectively. Total RNA was subsequently isolated and northern blot analysis of *SNCG* mRNA levels was performed.

Fig.1

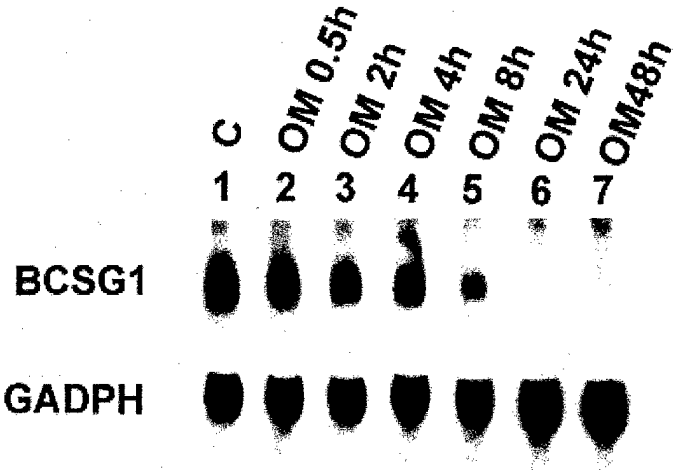


Fig.2A

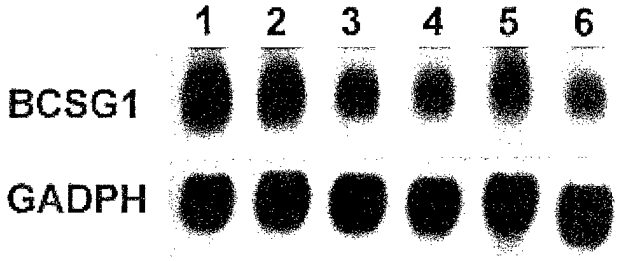


Fig.3

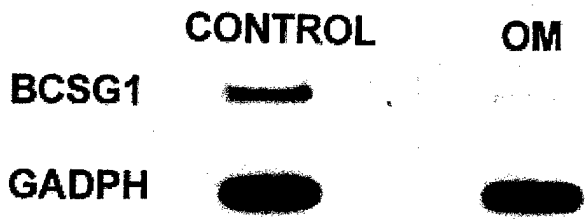


Figure 2B

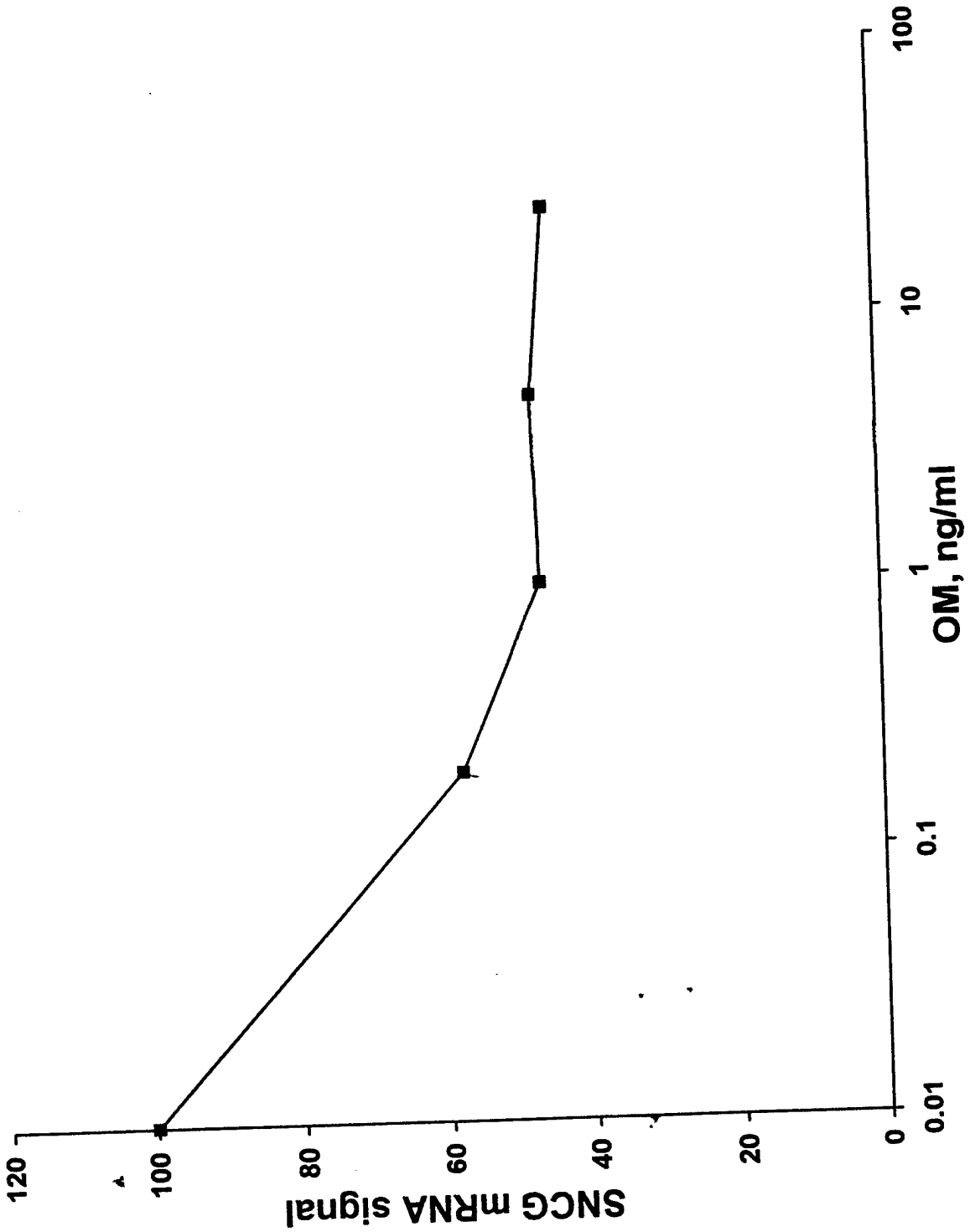


Fig.4

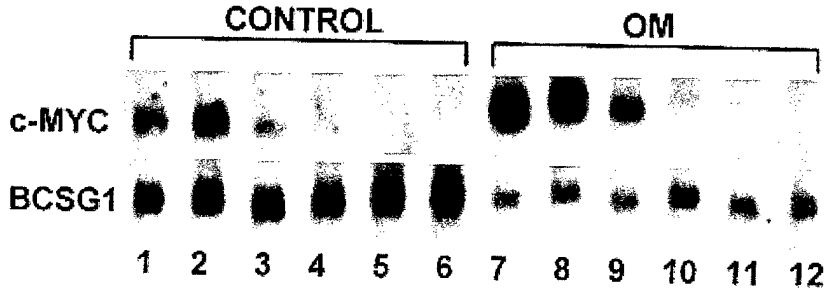


Fig.5

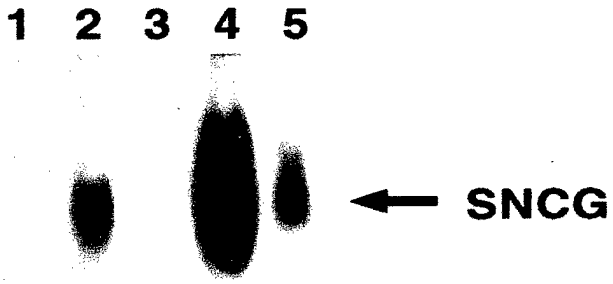


Fig.7

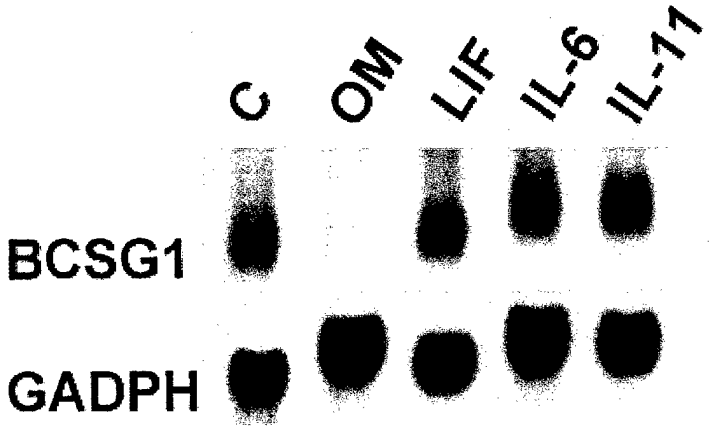
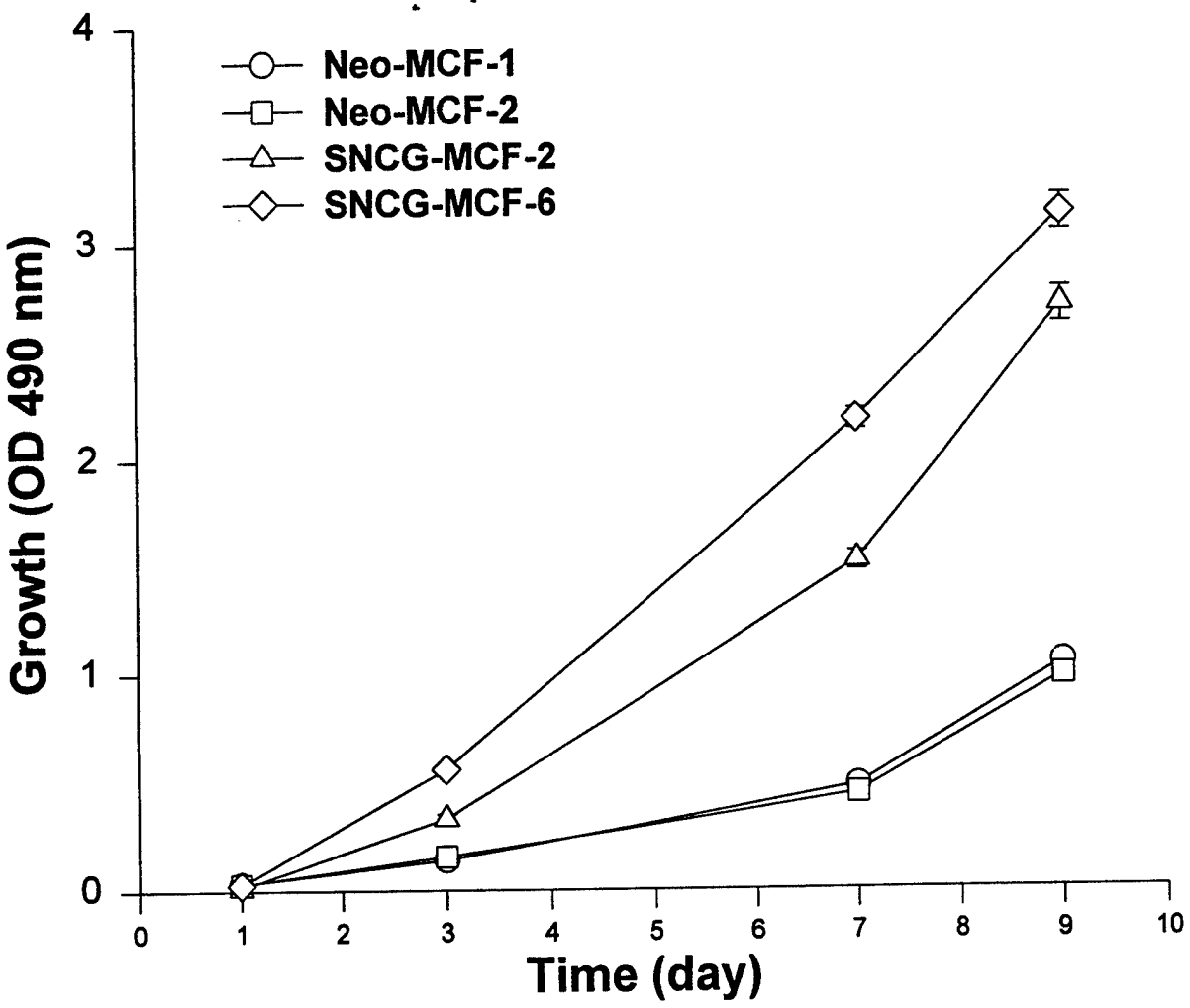


Figure 6


Neo-MCF-1
Neo-MCF-2
SNCG-MCF-2
SNCG-MCF-6
Growth (OD 490 nm)
Time (day)
0
1
2
3
4
0
1
2
3
4
5
6
7
8
9
10